

Analytical Profiles of Drug Substances

Volume 6

Edited by

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PREFACE

Although the official compendia list tests and limits for drug substances related to identity, purity, and strength, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. For drug substances important enough to be accorded monographs in the official compendia such supplemental information should also be made readily available. To this end the Pharmaceutical Analysis and Control Section, Academy of Pharmaceutical Sciences, has undertaken a cooperative venture to compile and publish Analytical Profiles of Drug Substances in a series of volumes of which this is the fifth.

The concept of analytical profiles is taking hold not only for compendial drugs but, increasingly, in the industrial research laboratories. Analytical profiles are being prepared and periodically updated to provide physicochemical and analytical information of new drug substances during the consecutive stages of research and development. Hopefully, then, in the not too distant future, the publication of an analytical profile will require a minimum of effort whenever a new drug substance is selected for compendial status.

The cooperative spirit of our contributors has made this venture possible. All those who have found the profiles useful are earnestly requested to contribute a monograph of their own. The editors stand ready to receive such contributions.

Klaus Florey

AMPHOTERICIN B

*Irvin M. Asher
George Schwartzman
and the
USASRG**

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The USASRG was formed at the request of P. Weiss, the National Center for Antibiotic Analysis, FDA, and is presently coordinated by the Office of Science, FDA. Individual contributions are referenced where possible.

TABLE OF CONTENTS

1. Description
 - 1.1 Drug Properties
 - 1.2 Chemical Properties
 - 1.3 The U.S. Standard
 - 1.4 Chemical Composition
 - 1.5 Structure
 - 1.6 Physical Description
2. Physical Properties
 - 2.1 Thermal Properties (DTA, TGA)
 - 2.2 X-Ray Powder Diffraction
 - 2.3 Solubility
 - 2.4 Acid-Base Properties
 - 2.5 Aggregation
3. Spectral Properties (Optical)
 - 3.1 Ultraviolet Absorption
 - 3.2 Infrared Absorption
 - 3.3 Raman Scattering
 - 3.4 ORD, CD, Specific Rotation
 - 3.5 Fluorescence
4. Spectral Properties (Other)
 - 4.1 Proton NMR
 - 4.2 ^{13}C -NMR
 - 4.3 Mass Spectrometry
5. Chromatography
 - 5.1 Paper
 - 5.2 Thin Layer
 - 5.3 High Pressure Liquid
 - 5.4 Gas
 - 5.5 Electrophoresis
6. Isolation
7. Stability
8. Antimicrobial Properties and Assays
9. Amphotericin A

1. DESCRIPTION

1.1 Drug Properties

Amphotericin B is a macrocyclic, polyene antibiotic produced by streptomyces nodosus (M4-575). It was originally isolated from a soil culture from the Orinoco River region, Venezuela (1). Used topically as a cream, or parenterally as a Na-desoxycholate suspension (Fungizone), it is effective against a broad variety of fungi and yeasts, and some protozoans (1-3; see Section 8).

The possibility that Amphotericin B combines with

cholesterol to form ion-transporting channels across cell membranes is being widely investigated (4-6). The absence of membrane sterols would thus explain the inability of Amphotericin B to affect bacterial growth.

In canine experiments (7), orally administered Amphotericin B induced a 20-45% reduction in serum cholesterol, suggesting a possible future role as a hypocholesterolemic agent. Amphotericin B has also been used (8) to treat canine prostatic hyperplasia (~ 30% reduction in gland size). However, the toxicity of the bile salt complex (9,10) may discourage such applications in humans. Work on less toxic derivatives is underway (3). In mice, intraperitoneal LD₅₀ is 280 mg/kg for Amphotericin B (3,11), 88 mg/kg for Fungizone and 1320 mg/kg for the methyl ester. The corresponding intravenous dosages are over an order of magnitude lower (3).

1.2 Chemical Properties

Amphotericin B is an amphoteric, macrocyclic heptaene with a mycosamine sugar head group. It yields a volatile base in concentrated NaOH and can bleach KMnO₄ or Br₂-CCl₄ (1). Its original separation was based on its solubility properties (1; see Section 6).

Amphotericin B is a particularly difficult antibiotic to characterize analytically. It is insoluble in many solvents (Section 2.3). Vibrator grinding dramatically affects X-ray powder diffraction patterns (Section 2.2) and infrared absorption spectra (Section 3.2).

pH dramatically affects ORD and specific rotation (Section 3.4). H₂O or CO₂ (or both) may be associated with the lattice (Section 1.4). Such contingencies have led to irreproducible results and conflicts in the literature. This report tries to analyze some of the pitfalls, but considerable caution (and often ingenuity) is still required for a meaningful analysis.

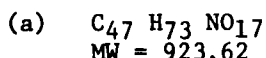
1.3 The U. S. Standard

The current U. S. antibiotic standard (Ampho. B-2; 11/27/74) was obtained from Squibb which markets the drug under the name Fungizone. The final stages of manufacture include precipitation from aqueous methanol (pH controlled by HCl then NaOH), washing with acetone, drying, and forcing through a sizing screen. The standard is stored in lots of 250 mg at -20°C, protected from light and moisture. Samples were dried for 3 hours at 60°C (\leq 5 mm pressure) before measuring potency, ultraviolet absorption, or specific rotation. There is also an Amphotericin B-1 (Amphotericin B-2 further recrystallized with various solvents and salts) for which no U. S. standard exists; it is not further

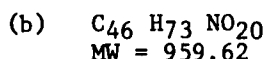
considered here. There is also an international standard (WHO) for Amphotericin B (12).

1.4 Chemical Composition

1.41 Empirical Formula and Molecular Weight
($^{12}\text{C} = 12.000$)



in agreement with recent x-ray (13) and mass spectrometric (14) measurements; accepted by USP-XIX (15), supersedes:



reported in Reference (11,16).

1.42 Elemental Composition

(a) $\text{C}_{47} \text{H}_{73} \text{NO}_{17}$ requires:

C 61.12%	H 7.96%	N 1.52%	O 29.45%
----------	---------	---------	----------

Reference 1 found:

C 60.40%	H 8.38%	N 1.62%	--
----------	---------	---------	----

with negative results for halogens, sulfur, and acetyl and methoxyl groups, for samples prepared by the methods of Reference 1.

(b) $\text{C}_{46} \text{H}_{73} \text{NO}_{20}$ requires:

C 57.58%	H 7.67%	N 1.46%	O 33.34%
----------	---------	---------	----------

and Reference 17 found:

C 57.17%	H 7.80%	N 1.20%	O 29.98%
----------	---------	---------	----------

for untreated U.S. standard Amphotericin B, consistent with the CHN results of References 18,19. (In the latter Amphotericin B was dried 3 hours at 80°C prior to analysis.) Other measurements (20) on dried samples of the U.S. standard (3 hours, 60°C) gave results (C 59.61%, H 8.32%, N 1.43%) closer to those of Reference 1.

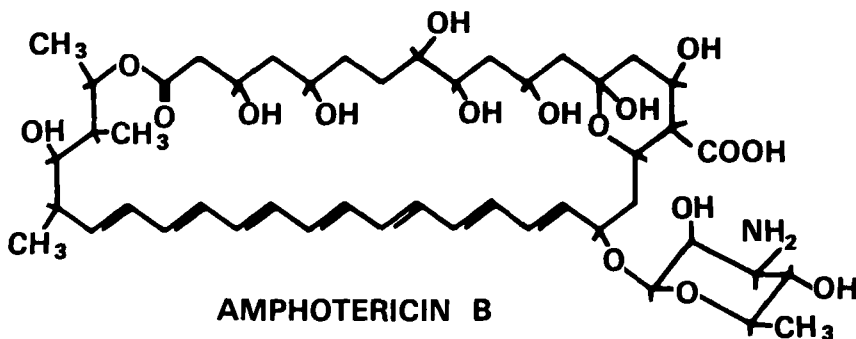
Notice that the oxygen content of Reference 17 is consistent with 1.41(a) rather than 1.41(b).

The full CHNO analysis of Reference 17 is consistent with the hydrochloride salt of 1.41(a) plus 1.5 waters of hydration. (Variation in water content alone can only partially resolve the discrepancies noted above.) However, tests (20) for Cl in the U.S. standard were negative ($\leq 0.11\%$).

(c) The Karl Fisher test gave 6.36% water content for the untreated U.S. standard (21). The standard exhibits a 4-5% loss on drying at 60°C under a vacuum. At atmospheric pressure, thermal gravimetric analysis (Section 2.12) indicates an $\sim 3.5\%$ weight loss between 60-100°C. Although some of this water may be adsorbed, some appears to be incorporated into the lattice; the Amphotericin B derivative investigated in Reference 13 incorporated three tetrahydrofuran molecules and one water molecule per unit cell.

1.5 Structure

The following structure is based on x-ray crystallographic studies of N-iodoacetyl Amphotericin B, tri-tetrahydrofuran monohydrate crystal (13). It corresponds to formula 1.41(a).



The rigid heptaene chain elongates the macrocycle, such that one side (polyene) is hydrophobic, while the other side (aliphatic) is hydrophilic due to the presence of seven hydroxyl groups and an ester carbonyl group. This may account for its ability to act as an ion-channel in membranes (4-6). A mycosamine residue is attached to one end, providing a free amino group. There is an internal hemi-ketal ring. It has been suggested (14) that the ketal-form may be in equilibrium with an open keto-form in solution. However, recent ^{13}C -NMR results (22) confirm the presence of the ketal-form in DMSO solution (Section 4.2), and provide no evidence for a keto-form in that environment.

This structure supersedes an earlier, partial structure by Cope, *et al.*, (23) which is incorrect in several details.

1.6 Physical Description

Bright yellow powder. Microscopic examination reveals prisms or needles for samples freshly recrystallized from dimethylformamide (11); but thin, irregular fragments (roughly 5-15 μ long, less than 0.3 μ thick) in the U.S. standard (25). The fragments tend to clump into large ($\sim 80 \mu$ diameter) clusters. The grinding process used in drug manufacture may also convert some crystals to an amorphous form (24; Section 2.2). A typical photomicrograph of the standard is shown in Figure 1.

2. PHYSICAL PROPERTIES

2.1 Thermal Properties

2.11 Differential Thermal Analysis (DTA)

DTA scans (25) show a gradual, approximately linear decrease from 35 to 135°C with peaks near 157 and 209°C (Figure 2). The sample begins to decompose above 200°C, without melting. The 157°C transition is accompanied by a change in color from bright yellow to brown-orange which begins around 130°C, and increases progressively. This presumably reflects an endothermic chemical change involving the chromophore.

2.12 Thermal Gravimetric Analysis (TGA)

TGA scans (25) show an $\sim 3.5\%$ weight loss starting below 65°C which reaches completion near 90°C (Figure 2). A further reduction in weight begins near 180°C and levels off near 220°C, with maximum slope near 205°C. These changes may reflect loss of residual solvent and decomposition respectively.

2.13 Melting Point

We find no evidence of the melting in Amphotericin B up to 250°C, at which temperature the antibiotic has already decomposed. This is consistent with Reference (1), but perhaps not Reference (16,18). Vaporization is detected (26) above 250°C in a mass spectrometer (vacuum $< 10^{-5}$ torr). Trimethylsilyl-ether derivatives of Amphotericin B may vaporize as low as 180°C (26).

2.2 X-Ray Powder Diffraction

The X-ray powder diffraction pattern of "untreated" (unground, unheated) U.S. standard Amphotericin B demonstrates definite crystalline structure. The observed d-spacings are given in Table 1 and Figure 3 (solid curve). Unground samples heated 15 minutes at 158°C produce a pattern with less intense peaks, slightly shifted d-spacings and increased background (Figure 3, dotted curve). These

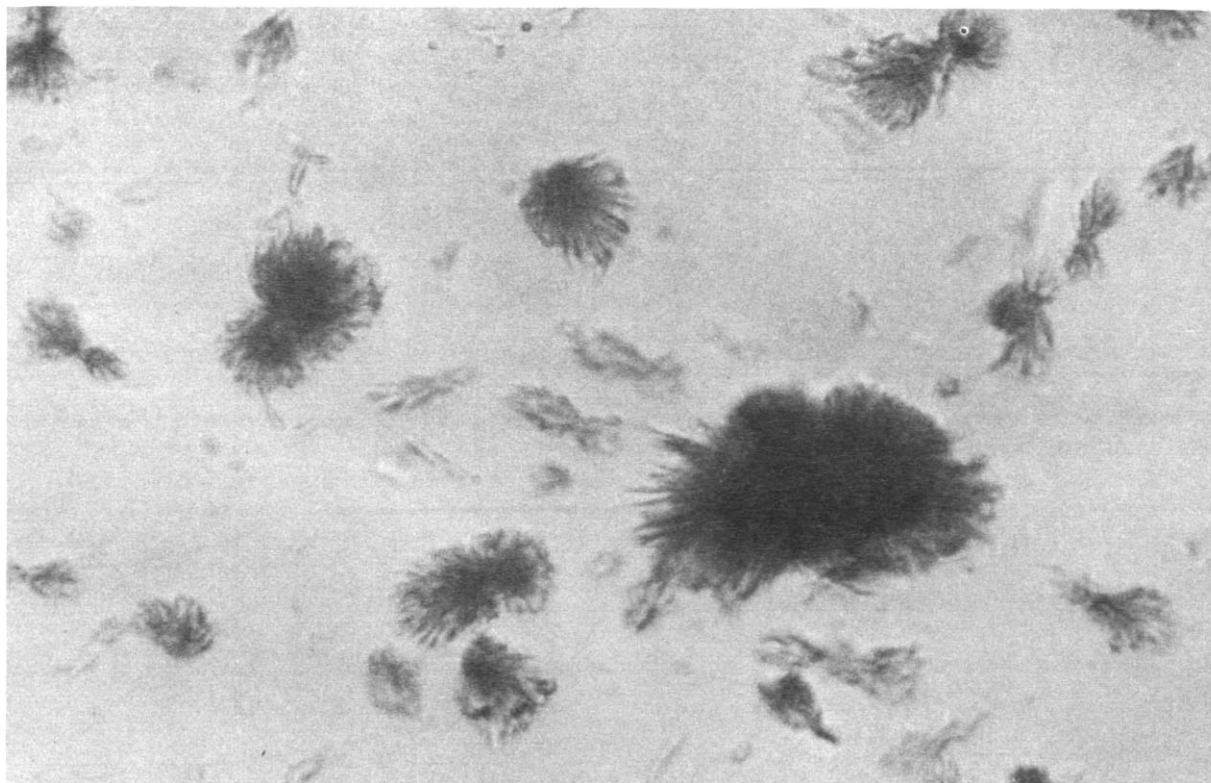


Figure 1. Photomicrograph (x100) of U.S. standard Amphotericin B. The final stages of the manufacturing process break the thin needles characteristic of the freshly recrystallized antibiotic.

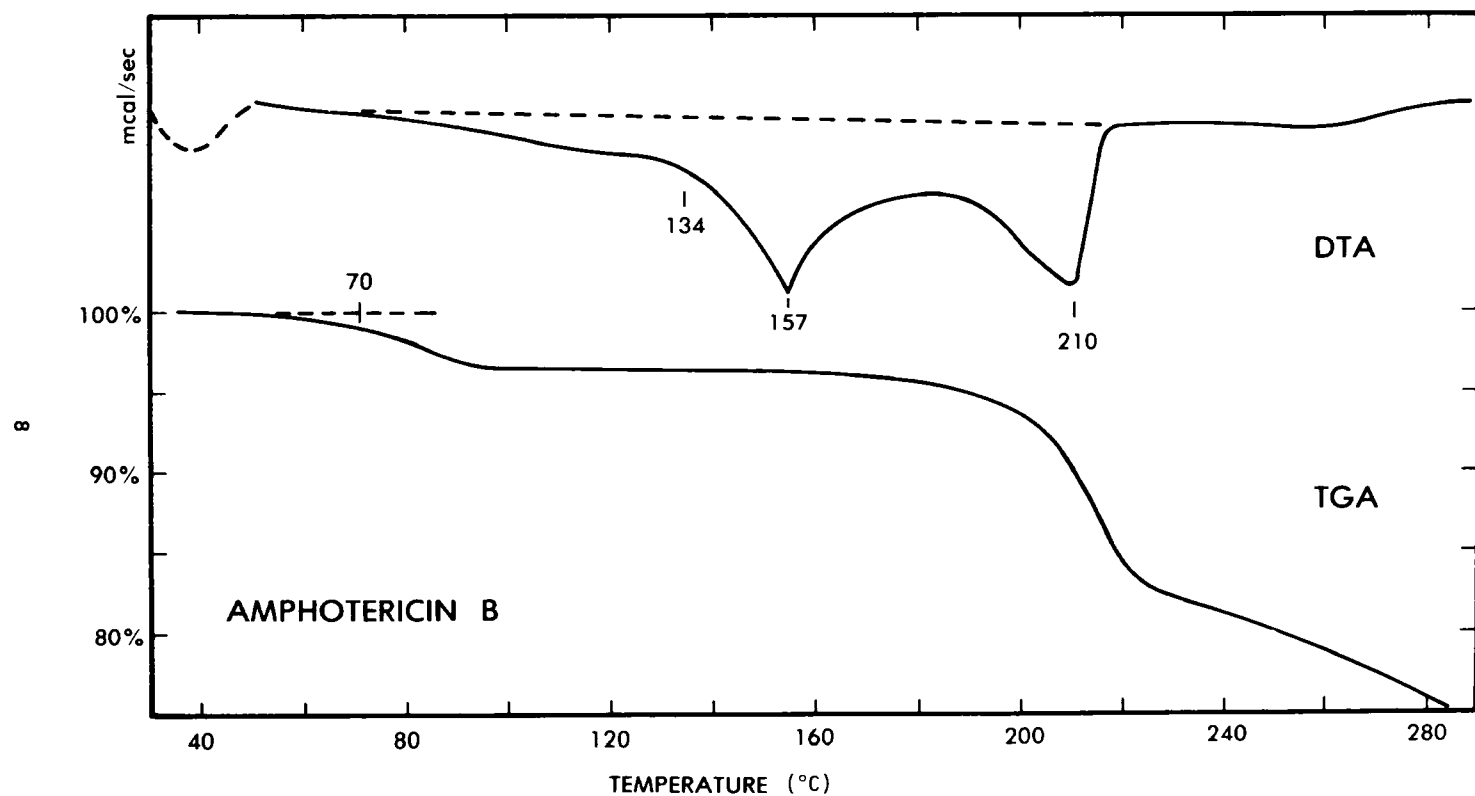


Figure 2. Differential thermal analysis (DTA) and thermal gravimetric analysis (TGA) scans of Amphotericin B.

TABLE 1
X-Ray Powder Diffraction Data
for Amphotericin B (Untreated Sample)

d(Å)	I/I ₀	d(Å)	I/I ₀
18.0	23	3.87	17
9.30	6	3.79	16
7.73	12	3.49	12
7.42	10	3.33	16
* 6.30	91	3.22	13
5.82	21	2.925 B	11
5.14	33	2.775	9
4.82	17	2.460 B	4
4.65	7	2.370	4
4.55	5	2.315 B	4
4.27	46	2.240 B	11
* 4.15	90	2.040 B	7
* 4.11	100		

T = triplet

B = broad

* = three most intense lines

TABLE 2
Solubility of Amphotericin B (MG/ML)

dimethyl sulfoxide (1)	30. - 40.
formamide	6.40
ethylene glycol	2.60
dimethyl formamide (1)	2. - 4.
acetic acid (1)	1. - 2.
propylene glycol (1)	1. - 2.
pyridine	1.75
methanol *	1.60
isoamyl alcohol	1.05
water	0.75
benzyl alcohol	0.75
1,4-dioxane	0.55
ethanol	0.50
ethyl ester	0.50
acetone	0.35
ethyl acetate	0.30
ethylene-Cl	0.30
isoamyl acetate	0.30
CS ₂	0.24
methyl ethyl ketone	0.16
isopr. alcohol	0.11
CHCl ₃	0.08
benzene	0.06
c-hexane	0.02
pet. ether	0.01
CCl ₄	0.002
toluene	0.0
iso-octane	0.0

*0.2 - 0.4 mg/ml for anhydrous methanol in Reference 1.

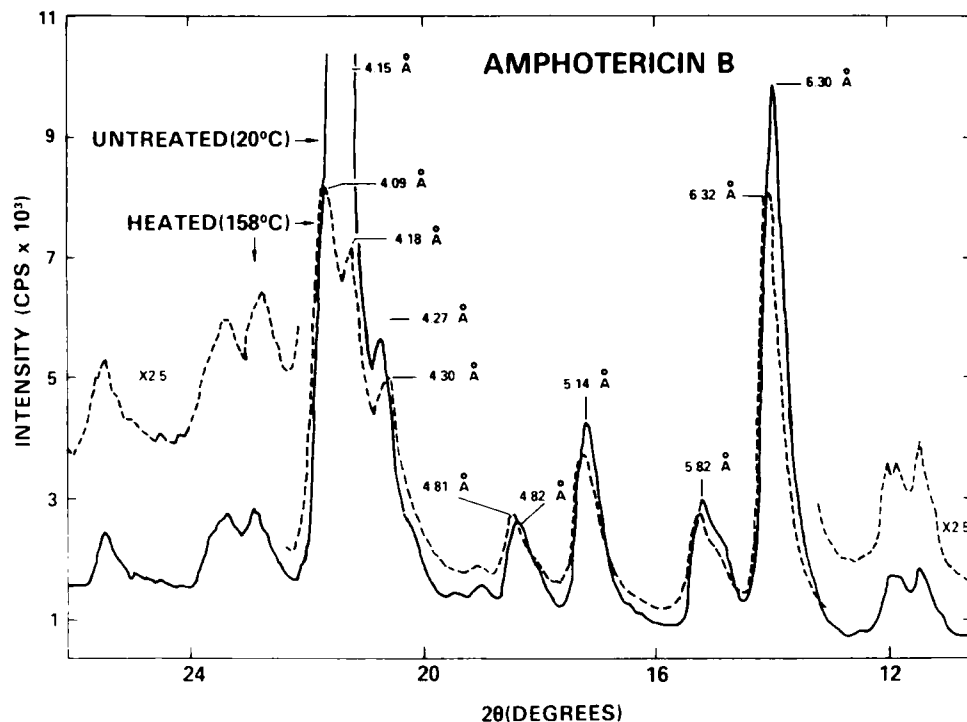


Figure 3. X-ray powder diffraction patterns of "untreated" (unheated, unground) Amphotericin B (—) and an aliquot heated to 158° C for 15 minutes (- - -). Both patterns taken at ambient temperature using a Philips wide-angle diffractometer equipped with a theta compensating slit and a focusing monochromator. The decreased peak intensities and elevated background of the heated material indicate some loss of crystallinity (~30%). Ordinate for the magnified (x2.5) insert is 4×10^2 cps.

changes indicate the introduction of additional strain in the crystal lattice and an increase in the amorphous (non-crystalline) fraction of the sample (24). Otherwise, the two patterns are highly similar.

In contrast, the diffraction pattern of vibrator-ground Amphotericin B (ground at room temperature in 2 mg. aliquots, 3 minutes each) displays only a few broad, weak peaks with a high background (Figure 4). Such a pattern is characteristic of amorphous powders, and demonstrates that the original crystalline powder has mostly undergone a transition to an amorphous form. This polymorphism explains the variations previously observed in infrared spectra (Section 3.2).

A complete structural determination of the N-iodoacetyl derivative (tri-tetrahydrofuran monohydrate crystal) is given in Reference 13 (see Section 1.5).

2.3 Solubility

As seen from its structure (Section 1.5), Amphotericin B is amphoteric with both polar (acidic and amino head groups) and nonpolar portions. It thus dissolves poorly in most pure solvents; exceptions are dimethylsulfoxide and dimethylformamide. The solubility data of Table 2, unless otherwise noted, are part of a previous FDA study (27).

Ionization of the acidic and amino groups often aids solvation (1,11):

	<u>H₂O</u>	<u>CH₃OH</u>	<u>dimethylformamide</u>
neutral	insoluble	0.2 - 0.4 mg/ml	2-4 mg/ml
acidic	0.1 mg/ml	3-5 mg/ml	60-80 mg/ml
basic	0.1 mg/ml	2-3 mg/ml	

Water solubility can be greatly increased by adding Na-lauryl sulfate (19) or Na-desoxycholate (as in commercial injectable Fungizone). Amphotericin B also dissolves in lecithin-cholesterol vesicles and sterol-containing natural membranes (4-6).

2.4 Acid-Base Properties

Titration (28) of 66% aqueous dimethylformamide solutions of Amphotericin B with methanolic HCl and KOH yields pK's near 5.7 and 10.0. Comparison with N-acetyl-Amphotericin B (pK=6.5) and Amphotericin B-methyl ester (pK=8.8) assigns the two pK's to carboxyl and amino groups respectively. Amphotericin B is found to be almost completely zwitterionic in this solution (tautomeric equilibrium

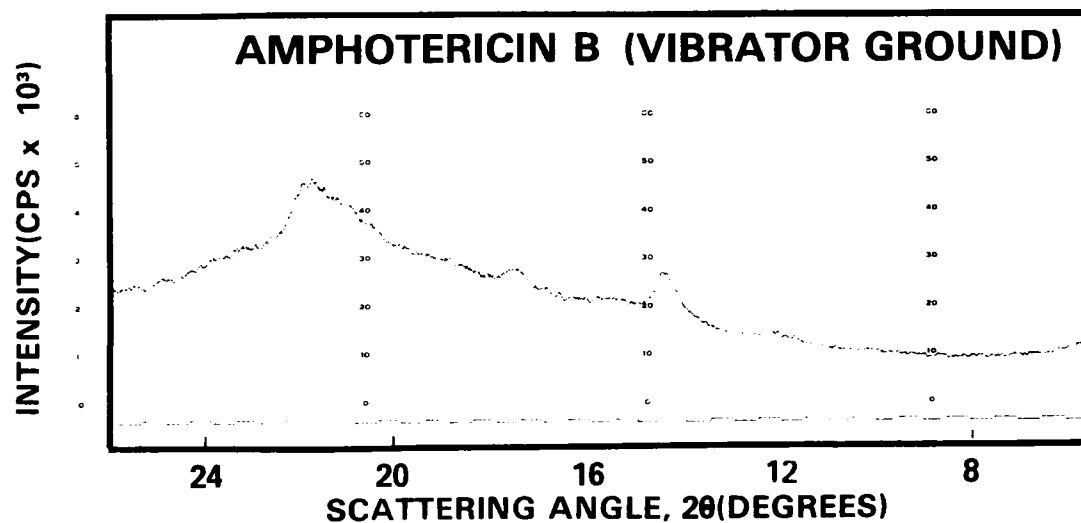


Figure 4. X-ray powder diffraction of Amphotericin B ground in a vibrator (3 min., 2 mg. at a time). The dramatic decrease in peak heights and increase in background demonstrate a phase transition to an amorphous form; little crystalline Amphotericin B remains.

constant $K_t = 1000$ with respect to the neutral molecule).

2.5 Aggregation

Measurements (29) of the ultraviolet absorption of aqueous solutions of Amphotericin B as a function of concentration do not obey the Beer-Lambert law. Subsequent Rayleigh light scattering measurements (29) indicate that Amphotericin B forms very large, labile aggregates of $\sim 2 \times 10^6$ M.W. in 10^{-4} - 10^{-5} M aqueous solutions (pH 7.9, in the presence of Na^+ -desoxycholate and phosphate). The aggregate mass is approximately unaffected by the addition of up to 35% $\text{C}_2\text{H}_5\text{OH}$, but drops precipitously thereafter. Similar effects are observed in the intensity of the 349, 367, 386, 409 nm ultraviolet absorption bands; however, the 328 nm band is affected by even 10% $\text{C}_2\text{H}_5\text{OH}$. The data are explained in terms of excitonic interactions between the heptaene chromophores of the aggregate. The aggregate mass was calculated using a (measured) value of 290. ml/mg for dn/dc , the change in the index of refraction with concentration of Amphotericin B.

3. SPECTRAL PROPERTIES (OPTICAL)

3.1 Ultraviolet

Amphotericin B has a highly characteristic ultraviolet absorption spectrum in DMSO, CH_3OH solutions (Figure 5). The sharp, intense bands arise from $\pi - \pi^*$ transitions of the heptaene chromophore. The same spectrum occurs in heated samples (15 minutes, 158°C), but with 25% less absorptivity. The intense 406, 382, 363, 345 nm. quartuplet of Amphotericin B shifts to 318, 304, 291, 289 nm. in Amphotericin A (1,18). Thus, an ultraviolet specification is part of the Federal Register (30) criteria of acceptability for Amphotericin B.

Spectra of Amphotericin B in aqueous solution (solubilized by DMSO or Na^+ -desoxycholate) are considerably different (Figure 6), and change further upon the addition of lecithin and/or cholesterol (31,32). These changes apparently reflect the presence of large, labile aggregates in such aqueous solutions (see Section 2.4). A more detailed account of Amphotericin B ultraviolet absorption spectra in various H_2O : $\text{C}_2\text{H}_5\text{OH}$ systems may be found in Reference (29). Ultraviolet reflection spectra of Amphotericin B monolayers on water yield three concentration-sensitive bands (33). The transition moment (oriented along the heptaene chain) lies within 6° of the water interface; the addition of cholesterol tilts this upward to approximately 35° .

3.2 Infrared

Literature spectra of Amphotericin B are contradictory (1,18,34). Two basic types of spectra are seen (Figures 7a,b). We find that both types can be obtained at

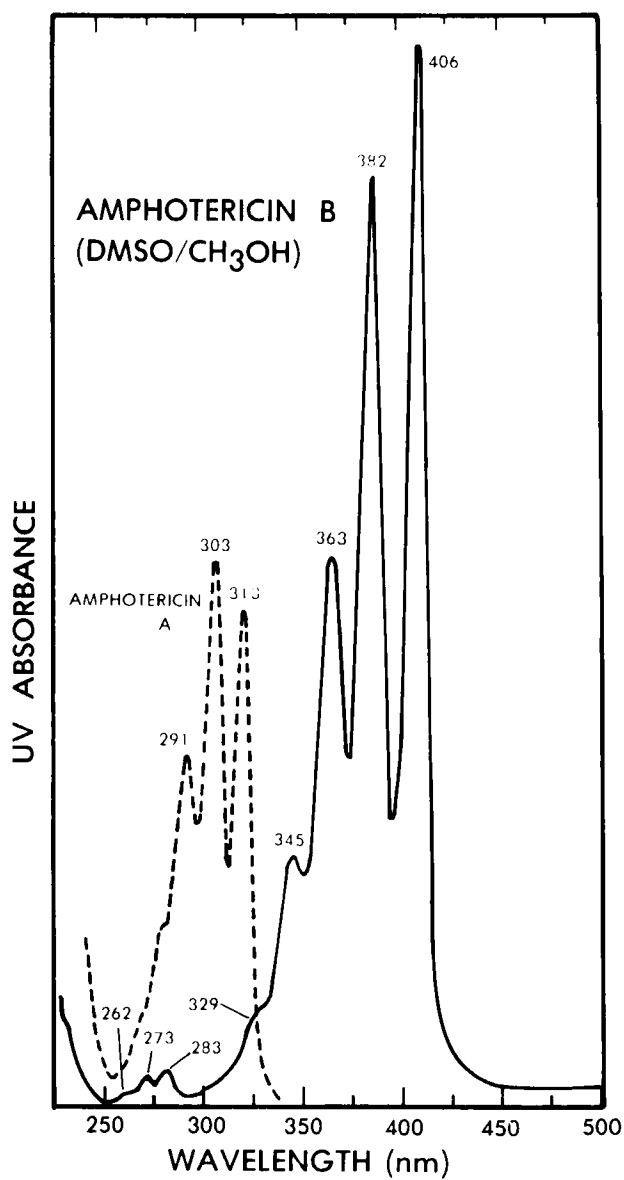


Figure 5. Ultraviolet absorption spectra of Amphotericins B and A in DMSO/CH₃OH solution (concentrations respectively 5.45, 8.32 $\mu\text{g/ml}$).

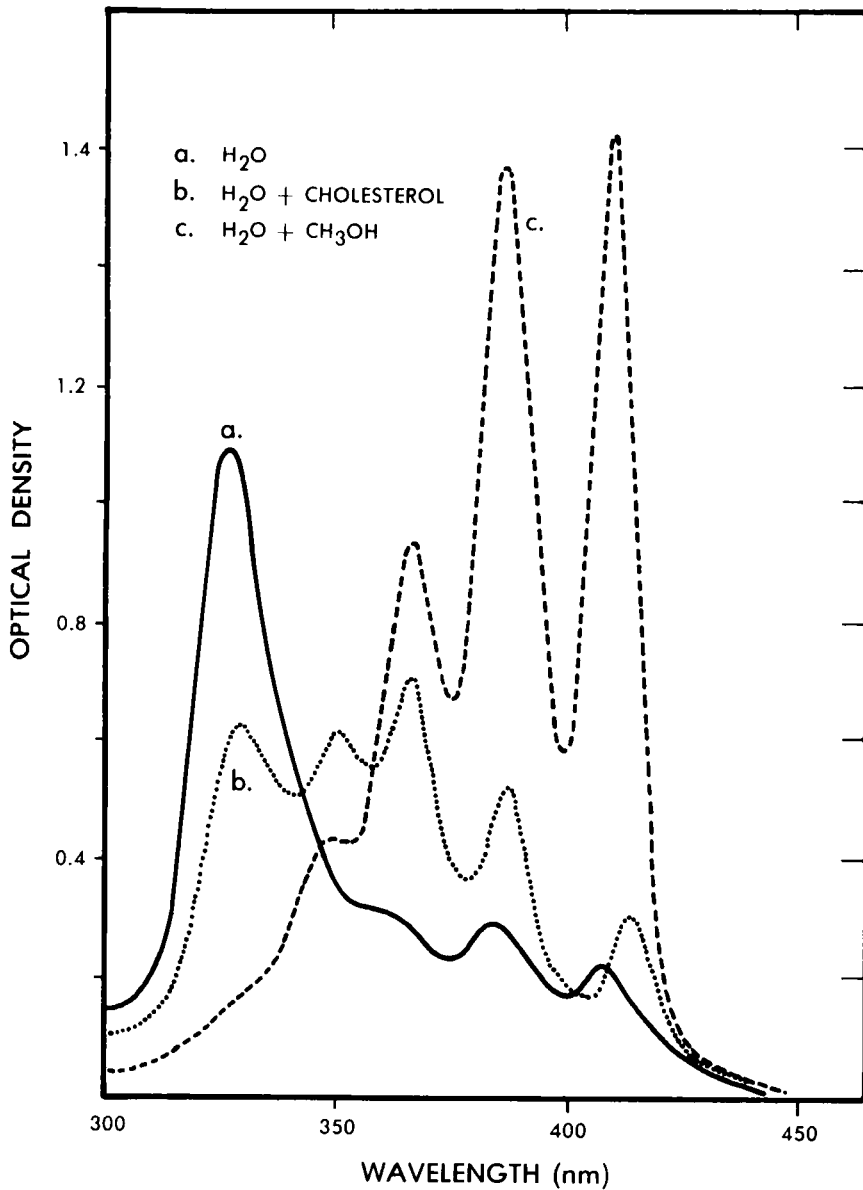


Figure 6. Ultraviolet absorption spectra of Amphotericin B (1 μM) solutions: (a) water, (b) water and cholesterol (10 μM), (c) water and methanol (1:1 v/v). (From Reference 32).

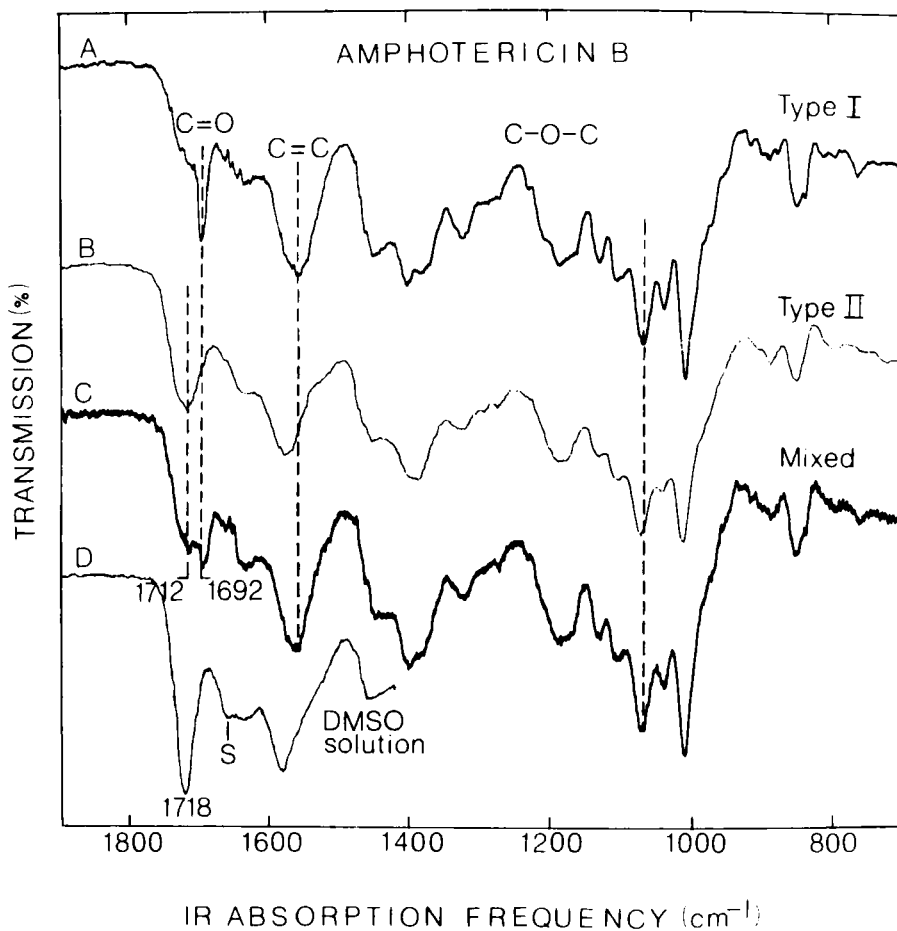


Figure 7. Infrared absorption spectra of Amphotericin B: (a) hand-ground powder, (b,c) vibrator ground powder pressed into KBr disks, (d) DMSO solution (saturated). Note the changes in the C=C and C=O stretch regions resulting from differences in sample preparation.

TABLE 3
Infrared Spectra

Type I	Type II	Tentative Assignment
(625)		
664		OH Out-of-plane Bend (?)
697		
(732)		
762	(sh)	Pyranose Ring Breathing (G)
795	(792)	
812	(804)sh	
818		
(837)sh		
851	850	} CH Bend (G)
(878)		
889	888	} CH Bend, CH ₃ Rock
(898)sh		
916		Pyranose Ring Vibration (G)
(931)sh		
(953)sh		
(972)sh		
(981)sh	(sl)	} CH Out-of-plane
1009	1010	
1041	1040	Bend (<u>trans</u> polyene)
1070	1068	
1109	1106	} CO Asym. Stretch (COC, COH)
1132	1130	
1164sh	(1173)B	
1186	(1188)B	COC Asym. Stretch (COC=O)
1210sh		
1233sh	(1230)sh	} CH ₂ Wag, Bend (skeletal)
1272sh	1269	
(sh)	(1291)	
1324	1322	
(1338)sh		
(1371)sh		
1381	(1385)B	} CH ₃ Sym. Bend, OH deformation
1401	(1400)B	
1448	1449	CH In-plane Bend (polyene)
1556*	1566*	CH ₂ ,CH ₃ Asym. Bend
(1628)B	1628sh	Polyene C=C Stretch
1692*		NH ₂ In-plane Bend
(1710)sh+	1712B*	} C=O Stretch
(sh)	2859*	
2918d	2859*	CH ₂ ,CH ₃ Symm. Stretch
	2925*	CH ₂ Asym. Stretch
2940d		
(2960)sh		CH ₃ Asym. Stretch
2978	(2979)sh	
3009	3015	CH Stretch (polyene)
(3370)		OH Stretch
3390B	3390B	(Strongly H-bonded)

NOTES:

B = broad, sh = shoulder, sl = slant, S = solvent peaks,
 () = weak, frequency uncertain, sym = symmetric, asym =
 asymmetric, * = frequency characteristic of Type I or Type
 II, and + = may arise from slight admixture of Type II.

room temperature, in the same medium (i.e., KBr pellet or Nujol mull) depending on the method of sample preparation (24). Handground powders typically yield type I spectra (Figure 5a; Reference 1,18), while vibrator ("wiggiebug") ground powders yield type II spectra (Figure 5b; Reference 34) or a more even mixture of the two types (Figure 7c).

Type I spectra are characterized by a sharp C=O stretch band at 1692 cm^{-1} , a 1556 cm^{-1} C=C stretch band and considerable substructure (e.g., $800\text{--}950\text{ cm}^{-1}$ region). Type II spectra are characterized by a broad C=O stretch band near 1712 cm^{-1} , a 1566 cm^{-1} C=C stretch band and less-resolved substructure. In "mixed" spectra (Figure 5c), superposition gives a C=O 1692 , 1710 cm^{-1} doublet. Spectra of DMSO solutions contain a C=O singlet near 1715 cm^{-1} .

X-ray powder diffraction studies (Section 2.2) show that type II spectra represent an amorphous phase induced by vibrator grinding (24); similar polymorphism has been observed in the Cinchona alkaloids (35). The broad shoulder observed near 1710 cm^{-1} in Figure 7a, may indicate an amorphous fraction in the standard (cf. 1.3). Hand-grinding of all samples would seem preferable in the future, especially when preceded by fresh recrystallization.

Heating the sample to 120°C has little effect on the spectrum. In contrast, the spectra of samples heated above the chemical transition near 157°C (Section 2.1) resemble Type II, even when handground. This is consistent with the $\sim 30\%$ increase in the amorphous fraction observed using x-ray powder diffraction (24).

The infrared absorption frequencies of Amphotericin B and their tentative identification are given in Table 3. Fourier transform infrared spectra confirm the existence of many of the weaker peaks. The 1692 cm^{-1} peak is actually a very close doublet.

The addition of Amphotericin B to aqueous suspensions of lecithin: cholesterol (3:1) vesicles shifts the midpoint of the "melting" transition of the lecithin sidechains from $\sim 41^{\circ}\text{C}$ to $\sim 45^{\circ}\text{C}$ (as monitored by frequency shifts in the CH stretch region; Reference 36). Because of the high infrared absorptivity of water, such measurements require the use of narrow, IRTAN sample cells.

3.3 RAMAN

Laser Raman spectra of Amphotericin B (37) are presented in Figure 8 and Table 4. The presence of a strong visible absorption resonantly enhances modes coupled to the chromophore. The intense peak near 1562 cm^{-1} corresponds to

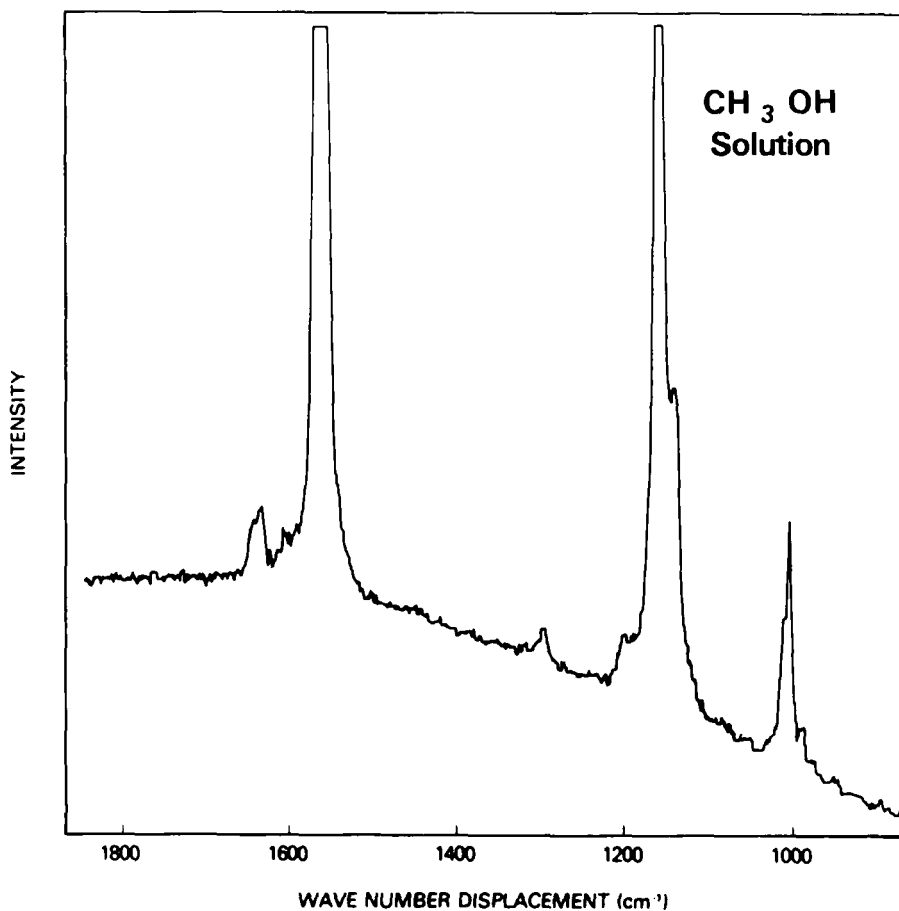


Figure 8. Resonance Raman spectra of Amphotericin B powder. Spectra taken with the 4880Å line of an Argon ion laser (incident power ~50 mw). Only those vibrations coupled to the polyene chromophore are enhanced sufficiently to be seen. There is a 4-fold increase in the intensity of the 1564 cm⁻¹ line upon changing from 514.5 nm to 457.9 nm.

TABLE 4
Resonance Raman Spectra (cm^{-1})

<u>Powder</u>	<u>CH₃OH</u>	<u>DMSO</u>	<u>Ref. (36)</u>	<u>KBr Pellet</u>	<u>Assignment</u>
922			(980)		C=CC, HCC in-plane Bend
			995		
1007 (1014)sh		1011		1007	
1142	1136sh	1140sh	1131sh	1136sh	CC Stretch, HCC In-plane Bend (mixed with C=C Stretch)
1159	1156	1161	1152	1156	
1202	1198	1201	(1195)	(1198)	
1298	(1298)		1287		
1562	1559	1562	1554	1559	C=C Stretch (intense)
1608	1602	1607	(1597)		
			1624		
1635	1639	1640	1636	(B)	C=O Stretch (mixed with C=C Stretch)
(1645)sh				(B)	
	(1666)	(1661)			

almost pure C=C stretch, whereas the weak 1635-1645 cm^{-1} modes also contain considerable C=O stretch contributions. However, the numerous nonresonant modes could not be observed, even using a dye laser. Notice that several of the Raman modes are not infrared active (compare Section 3.2).

Solid-state spectra differ only slightly from those in CH_3OH or DMSO solution (37). However, our results differ markedly from previous observations of wet Amphotericin B powder smeared on filter paper (38); in particular, we observe a peak near 1010 cm^{-1} . The supposed absence of a 1010 cm^{-1} Amphotericin peak in previous spectra was used to interpret carotenoid spectra (38).

Spectra of heated Amphotericin B powder (15 minutes at 158°C) dissolved in CH_3OH (pH 5.) appear normal, despite the change in sample color (Section 2.1). However, lowering the pH to <1 causes immediate decomposition into a product in which the intensity of the prominent 1156, 1559 cm^{-1} peaks is markedly reduced.

3.4 ORD, CD, Specific Rotation

The specific rotation, $[\alpha]_D^{24\text{C}}$ of Amphotericin B has been given as -33.6° and $+333^\circ$ in 0.1N methanolic HCl and "acidic" DMF respectively (1,11). However, closer investigation (39) shows that the specific rotation is highly pH dependent. It is approximately $+285$ and pH 1.0, and $+413$ at pH 2.1, in DMF (2.5 mg/ml). (The "pH" was measured with a Beckman pH-meter with one glass and one KCl electrode).

Circular dichroism (CD) spectra of Amphotericin B in H_2O , $\text{CH}_3\text{OH}/\text{H}_2\text{O}$, and $\text{H}_2\text{O}/\text{cholesterol}$ (32) are given in Figure 9. The corresponding optical rotatory dispersion (ORD) spectra in CH_3OH (0.1N HCl) and DMF (pH 2.2) solutions (40) are given in Figure 10.

All CD peaks in $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ closely match Amphotericin B ultraviolet absorption frequencies; the peak rotations are positive for the strong 340-420 nm. quadruplet, and negative for the weak 260-290 nm. triplet (Figure 9c). The CD spectra of DMSO-solubilized Amphotericin B in H_2O and $\text{H}_2\text{O}/\text{cholesterol}$ are less complex, opposite in sign and an order of magnitude more intense. Preparations of Squibb Fungizone (Amphotericin B solubilized in H_2O by Na^+ -desoxycholate) are similar but even more optically active (Figure 9 a, b).

The optical rotation in acidic CH_3OH (40) displays appreciable changes only in the 260-300 nm region, whereas in acidic DMF both regions show considerable changes. In acidic DMF, the rotation near 271, 392, 413 nm. is positive and the maximum near 290 nm. becomes a minimum (Figure 10). ORD measurements (41) in neutral CH_3OH somewhat resemble those in acidic DMF; however, the 286 nm. band is assigned to an

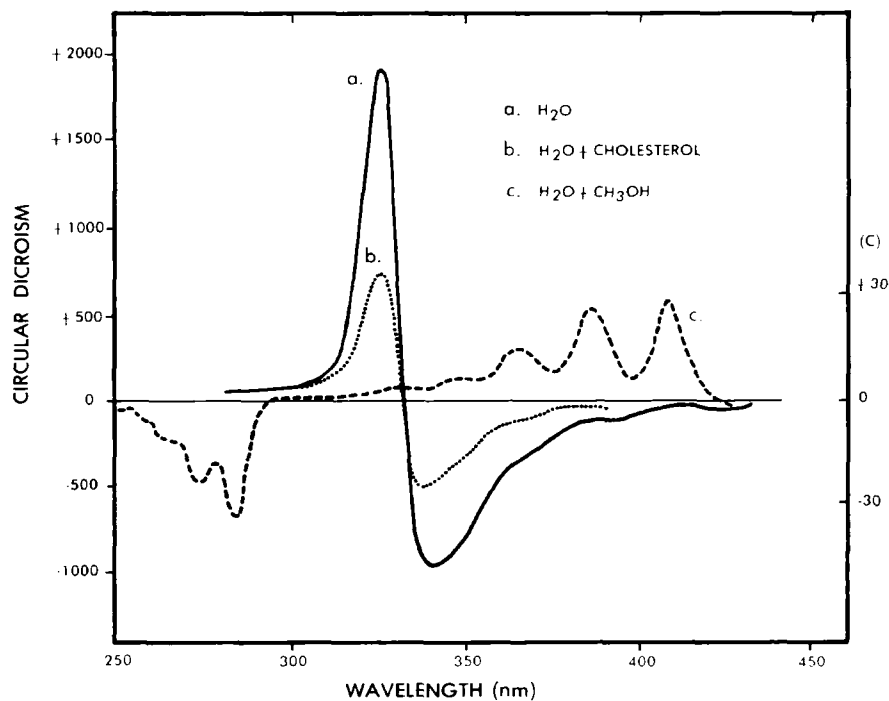


Figure 9. Circular dichroism (CD) spectra of Amphotericin B (1 μM) solutions: (a) water, (b) water and cholesterol (10 μM), and (c) water and methanol (1:1 v/v). (32b) Preparations of Squibb Fungizone (Amphotericin B solubilized in H₂O by Na⁺-desoxycholate) are similar but even more optically active (Figure 9 a,b).

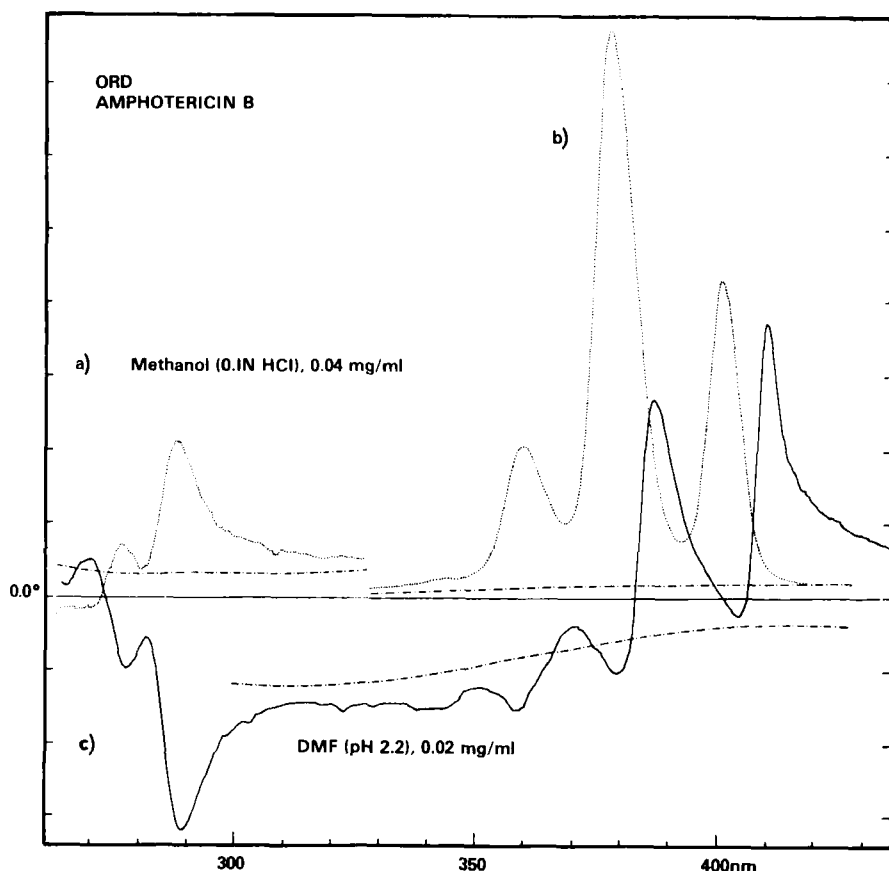


Figure 10. Optical rotatory dispersion (ORD) of Amphotericin B in acidic methanol (a,b) and acidic DMF (c) with base lines (---). Vertical units are (a) 0.01° , (b) 0.04° , (c) 0.10° . There may be some spectral change in the 20 minute interval required to obtain the spectrum (a,b). Amphotericin B undergoes a chemical change in 0.1N HCl-methanol (40). The optical rotation appears to be $+87.7^\circ$ soon after dissolution (0.2 mg/ml), but decreases approximately linearly from $+80.5$ to -30.2° in 12 minutes in another experiment (2.0 mg/ml). Thus, the values given in References 1,11 should be viewed with caution.

impurity. Reduction with Na-borohydride has little effect on the ORD spectra, suggesting the absence of the ketone (and presence of the hemi-ketal) form in neutral methanol.

3.5 Fluorescence

The fluorescence spectrum of Amphotericin B (8.35 μ M in saline Tris buffer) is greatly enhanced by incorporation into lecithin vesicles (31). This effect is substantially reduced in the presence of epicholesterol but not cholesterol or ergosterol. The fluorescence emission for 340 nm excitation is considerable between 410-500 nm, with broad maxima near 427, 451, 472 nm. The most effective excitation wavelengths for 480 nm emission lie between 300-345 nm, with broad maxima near 310, 333 nm (31). In free aqueous solution (10 μ M, 50°C) the addition of cholesterol slightly lowers the partial quantum efficiency (355 nm excitation, 475 nm detection; Reference 42).

4. SPECTRAL PROPERTIES (OTHER)

4.1 Proton NMR

A typical 60 MHz proton NMR spectrum of Amphotericin B in DMSO- d_6 solution (43) is presented in Figure 11a. The broad signals can only be loosely identified with specific chemical groups. Substructure is present (c.f. the 1.19 ppm broad multiplet) but difficult to resolve in the 60 MHz spectrum.

Amphotericin B has 13 exchangeable protons (10 hydroxyl, 2 amino, 1 acid). Rapid exchange between H₂O and Amphotericin protons gives rise to a combined OH singlet. Its position is highly variable and depends upon the extent of Amphotericin-H₂O hydrogen bonding, and thus H₂O concentration. Positions between 3.8 and 4.7 ppm are typical (19, 43).

The 220 MHz spectrum (Figure 11b) resolved considerable detail (e.g., more than 10 resonant signals between 0.7 - 1.7 ppm), although the complexity of the molecule makes detailed assignments difficult (44).

4.2 ¹³C-NMR

¹³C-NMR spectra of Amphotericin B and its N-acetyl and methyl ester derivatives clearly demonstrate the presence of a hemi-ketal ring in DMSO- d_6 solution (22) consistent with the solid-state conformation of Reference 13. There is no evidence of an equilibrium with a keto-form. In un-derivatized Amphotericin B, the hemi-ketal and hemi-acetal (mycosamine C-1) carbons appear at 97.1 and 95.9 ppm respectively; they are respectively a singlet and a doublet in off-resonance measurements. The lactone and COO⁻ carbonyl

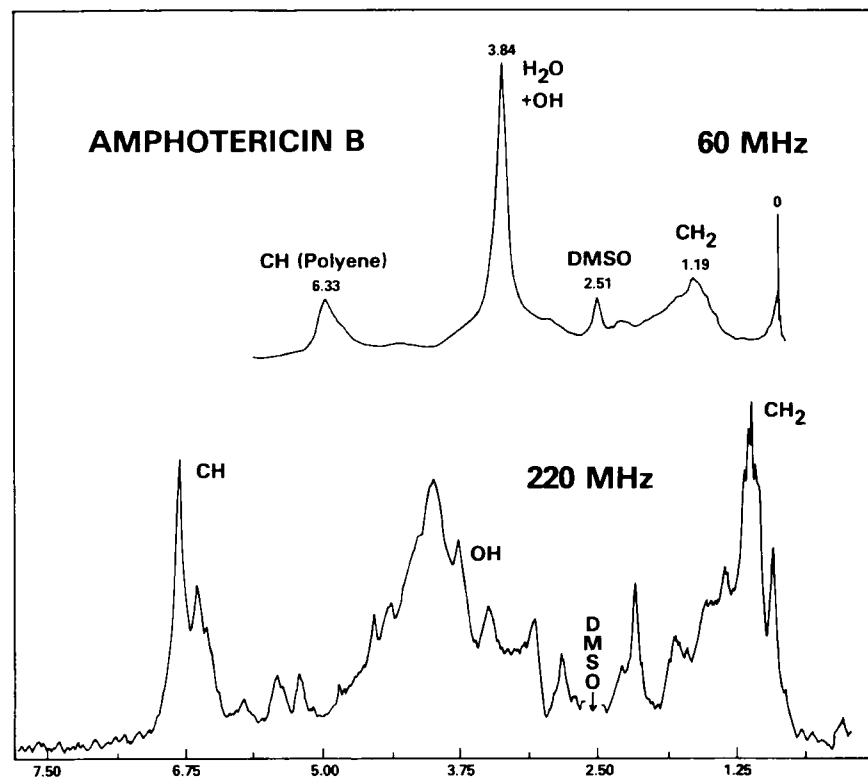


Figure 11. 60 MHz and 200 MHz proton nuclear magnetic resonance spectra of Amphotericin B in d_6 -DMSO. The complex substructure can be resolved in the latter.

carbons appear at 170.6, 177.6 ppm respectively. A typical spectrum of the U.S. standard (45) appears in Figure 12.

4.3 Mass Spectrometry

Early mass spectrometric attempts at structural elucidation were not completely successful (23). More recent studies (14; photo plate detector) of the per-TMS and per-d₉-TMS derivatives are consistent with structure 1.5

(TMS = trimethyl-saline). The fragmentation pattern of Amphotericin B is far more complex than that of nystatin, despite their close chemical resemblance. Additional mass spectra (46; electrical detector calibrated to m/e 1800) of the TMS-ether derivative are presented in Table 5. Despite general agreement several characteristic ions differ by 1-2 amu, or are not observed (Table 6).

The M-150 fragment (m/e 1637) represents the loss of CO₂ CH₃, and TMS:OH from the molecular ion; fragments f, g, h, i represent the loss of additional TMS:OH. Fragment l (m/e 1346) represents M-150 minus a doubly substituted mycosamine fragment (m/e 201). Further losses of TMS:OH from fragment l yield fragments m, n, o, q, r.

The glycoside linkage is particularly vulnerable to fragmentation (46). The triply TMS-substituted mycosamine-ester fragment gives rise to an intense m/e 362 (80.5%) peak; charge retention on the opposite side of the linkage was less common (m/e 378, 4.05%). No sugar fragments were found with all four labile hydrogens replaced (m/e 434, 450).

5. CHROMATOGRAPHY

5.1 Paper

The original method (1) utilized Whatman No. 1 paper pretreated with 0.3M K₃PO₄ buffer (pH 3.0). Spot developed 6-7 hours with 80% propanol. The mobility was R_f(B) = 0.5 for Amphotericin B and R_f(A) = 0.7 for Amphotericin A. However, the low pH damaged the antibiotics, preventing longer development. High-pressure liquid techniques (Section 5.3) are preferable for automation, quantitation, and collection.

Alternate methods (51) utilize Whatman No. 1 paper pretreated with McIlvaine's buffer, equilibrated over solvent for 1 hour, and developed for 5 hours. The results are:

<u>Solvents</u>	<u>R_f(A)</u>	<u>R_f(B)</u>	<u>pH</u>	<u>T(°C)</u>
Sec-butanol: H ₂ O: CaCl ₂ (20 ml: 80 ml: 200 mg)	0.82	0.64	3.2	37

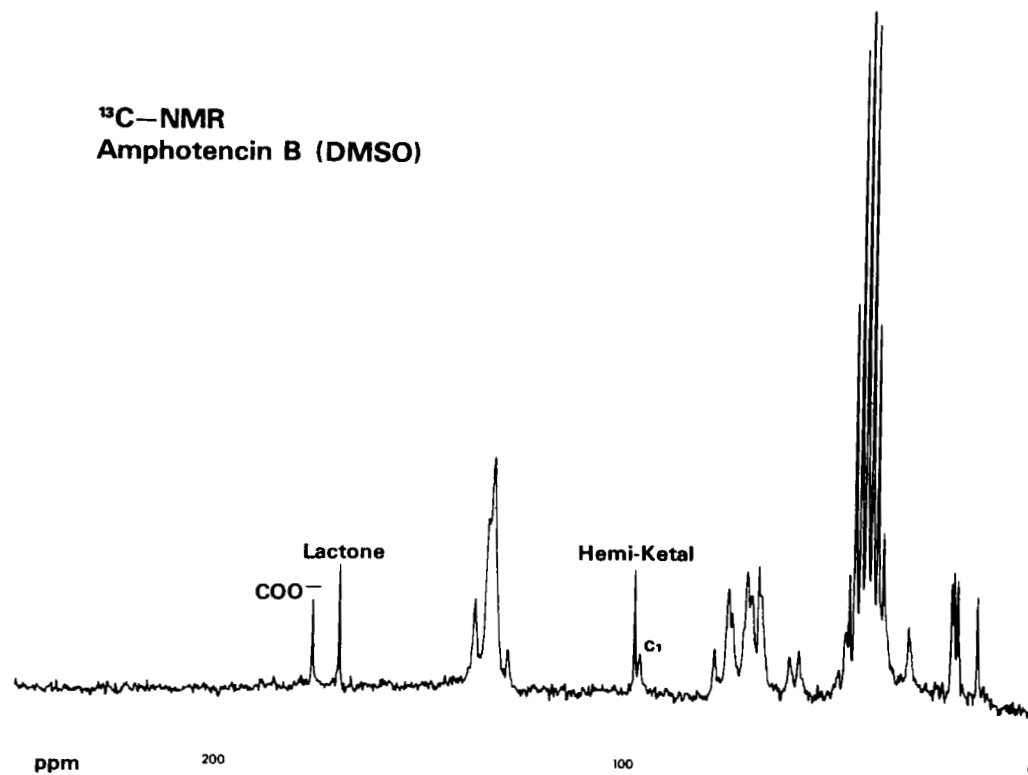


Figure 12. ^{13}C -NMR spectrum of Amphotericin B in DMSO-d_6 solution (saturated).

TABLE 5
High Mass Portion of the Spectrum of
Amphotericin B-TMSI

<u>I/BASE</u>	<u>MASS</u>	<u>I/BASE</u>	<u>MASS</u>
0.74%	706.5	0.11%	791.5
0.14%	707.3	0.71%	793.5
0.51%	708.4	1.30%	794.3
1.44%	711.3	1.33%	796.3
1.40%	715.6	0.24%	798.7
1.75%	716.4	0.87%	804.3
0.96%	720.5	0.41%	805.5
0.76%	722.3	3.63%	806.6
3.72%	723.5	2.83%	807.3
0.89%	724.2	2.64%	810.5
3.16%	726.1	0.29%	811.3
0.09%	729.8	1.05%	813.7
1.04%	731.5	0.58%	815.5
1.56%	734.5	0.87%	817.1
2.70%	735.4	3.23%	818.2
3.14%	737.6	2.71%	819.6
0.63%	738.5	0.21%	820.3
3.11%	741.4	1.05%	823.3
1.15%	745.3	2.08%	826.5
0.61%	746.3	3.24%	835.2
1.08%	747.7	2.52%	836.1
0.31%	749.3	2.05%	837.2
0.50%	751.8	1.96%	838.4
1.88%	754.5	1.23%	839.3
0.16%	756.7	0.18%	840.4
1.04%	760.3	0.53%	841.1
2.88%	761.3	0.40%	844.0
0.09%	762.7	0.71%	846.8
1.61%	763.8	1.36%	848.5
0.80%	765.2	1.76%	851.0
0.36%	766.4	0.71%	852.0
2.28%	768.7	0.92%	853.2
1.27%	769.3	0.56%	857.0
1.28%	770.8	2.53%	861.5
0.98%	771.3	0.56%	865.4
0.95%	773.0	1.50%	866.3
2.29%	777.5	0.28%	867.5
1.61%	778.4	0.76%	868.3
1.90%	781.6	0.16%	868.9
1.26%	782.5	0.51%	869.6
0.74%	785.5	1.96%	877.0
1.05%	788.8	2.79%	881.3
0.61%	790.2	1.52%	882.4

0.44%	884.5	1.32%	1056.9
1.84%	888.8	1.56%	1058.1
1.23%	890.8	0.13%	1059.9
0.66%	891.3	0.31%	1061.2
0.31%	892.2	0.50%	1064.6
0.31%	893.1	0.77%	1072.0
1.29%	894.5	1.24%	1076.5
1.31%	897.9	1.21%	1094.3
1.06%	899.3	0.50%	1110.1
2.95%	907.4	0.06%	1122.1
0.09%	908.4	0.42%	1123.1
0.57%	910.4	0.79%	1134.0
0.74%	912.7	0.99%	1148.5
0.74%	916.3	0.95%	1151.3
0.61%	918.6	0.40%	1153.1
0.74%	921.1	0.12%	1155.6
1.12%	922.6	0.26%	1171.3
1.30%	924.3	0.78%	1178.2
1.40%	933.1	2.04%	1204.3
3.25%	936.4	1.07%	1207.7
0.40%	943.0	0.06%	1209.3
1.03%	943.8	0.56%	1216.7
2.34%	952.5	1.26%	1223.0
0.50%	954.0	2.08%	1226.5
0.62%	957.8	2.11%	1228.6
1.65%	960.8	0.47%	1229.7
1.98%	965.8	0.53%	1232.8
0.18%	967.5	0.37%	1241.8
1.25%	969.4	0.38%	1247.6
0.20%	974.1	0.44%	1249.8
0.33%	976.1	0.51%	1250.6
0.96%	978.1	1.51%	1255.8
0.17%	980.2	1.33%	1257.3
1.57%	982.9	0.81%	1260.4
0.08%	985.6	0.93%	1268.0
0.64%	987.7	0.45%	1278.0
2.12%	993.4	0.67%	1280.5
0.16%	1000.8	0.45%	1293.2
0.09%	1003.2	0.75%	1300.5
0.83%	1004.8	0.21%	1312.9
1.63%	1006.3	0.28%	1319.1
1.61%	1016.3	0.26%	1323.8
1.55%	1019.1	0.90%	1332.0
0.48%	1024.0	0.52%	1334.8
0.69%	1041.2	0.37%	1340.8
1.04%	1044.8	0.64%	1345.6
2.21%	1046.6	0.67%	1351.5
1.67%	1050.3	1.84%	1363.5
1.32%	1056.9	1.77%	1366.4

0.13%	1360.5
0.94%	1374.0
0.35%	1393.9
1.00%	1406.5
0.37%	1412.8
1.01%	1417.5
0.72%	1423.6
0.84%	1431.1
1.41%	1433.1
0.73%	1441.8
0.05%	1445.0
0.13%	1449.5
0.48%	1451.3
0.98%	1455.5
1.25%	1491.1
0.36%	1499.1
0.13%	1500.8
0.35%	1516.8
0.29%	1532.4
0.41%	1539.3
2.06%	1549.3
1.57%	1572.8
1.82%	1594.5
0.93%	1607.9
0.21%	1641.2
0.43%	1650.5
0.07%	1652.5

TABLE 6
Comparison of Characteristic Ions of
Amphotericin B-TMSi

Reference 14		Reference 46	
	m/e	m/e	% R. Intensity
M+	1787		
M-TMSi	1714		
M-150(e)	1637		Not Observed
	1624		Not Observed
(f)	1547	1549.3*	2.0
	1534	1532.4*	0.3
(g)	1457	1455.5*	0.9
	1444	1445.0*	0.05
(h)	1367	1366.4	1.8
(l)	1346	1345.6	0.6
(i)	1277	1278.0*	0.5
(m)	1256	1255.8	1.5
(n)	1166		Not Observed
(o)	1076	1076.5	1.2
(j)	988	987.7	0.6
(q)	986	985.6	0.1
(p)	896	897.9*	1.3
(r)	806	806.6	3.6
(k)	718		Not Observed

* Measurements differ by ≥ 1 amu.

Same (paper not equilibrated)	0.86	0.41	3.2	37
Acetone: H ₂ O (8:2)	0.77	0.59	4.6	25

The location of the antibiotics was determined by bioautography using Candida tropicalis (SC 1647), using the method for nystatin (52).

5.2 Thin Layer (TLC)

Most usable solvent systems for thin-layer chromatography (TLC) of Amphotericin B contain alcohol (Table 6). Solvent system G should separate Amphotericin B (R_f 0.32) from Amphotericin A. Solvent systems G,J should separate Amphotericin B (R_f = 0.32, 0.18 respectively) from nystatin (R_f 0.65, 0.54 respectively). Other references are found in Reference 3.

5.3 High-Pressure Liquid (HPLC)

Using a Waters Associates (Milford, Mass.) μ C18 column, high-pressure liquid chromatography (HPLC) could separate solutions of Amphotericin B from small amounts of an accompanying degradation product in a variety of acidic methanol systems. The contaminant ranged from 0.7% in fresh solutions to ~3% in old solutions using the solvent systems of Reference 53.

The useful separation of Amphotericin A and B is more difficult, but can be achieved using the following procedure (53): 20% CH₃OH/80% DMF to 100% CH₃OH over 5 minutes, straight or concave gradient, 1.5 ml/min flow, absorption monitored at 280 nm. Separation requires less than 20 minutes. Maximum resolution (narrowest peaks) was obtained for a concave gradient (Figure 12). Separation was not achieved in CH₃OH, despite earlier reports of success with less efficient columns (54). The B/A ultraviolet absorbance ratio is 0.6 near 280 nm.

The retention times found by other workers (55) using VYDAC-RP (30-44 μ m) columns with H₂O:CH₃OH:tetrahydrofuran (420:90:45) for Amphotericin B (3.4 minutes) and nystatin (3.0, 3.4 minutes) are too similar to differentiate between them. The method of Reference 53 is also unable to separate Amphotericin B and nystatin.

5.4 Gas

Controlled pyrolysis followed by gas chromatography of the resulting fragments (> 30) gave distinct "fingerprints" for nystatin and Amphotericin B (56).

5.5 Electrophoresis

Electrophoretic mobilities of Amphotericin B,

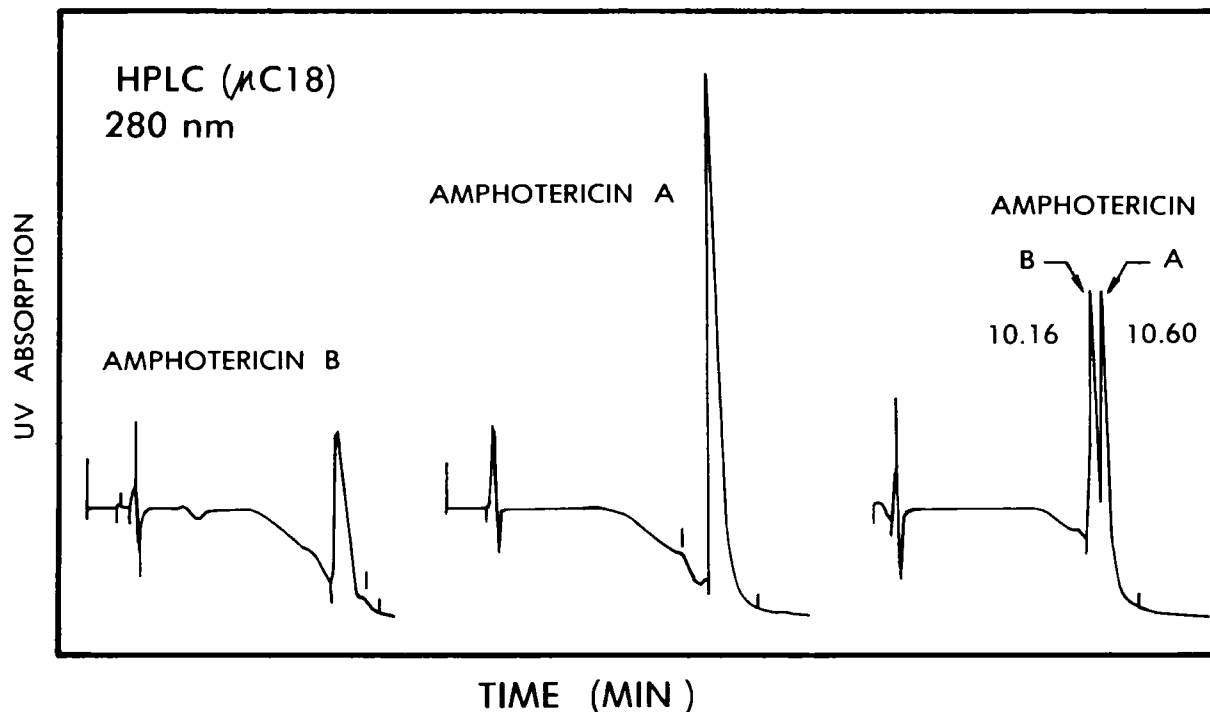


Figure 13. High-pressure liquid chromatograms of: (a) Amphotericin B dissolved in acidic methanol (1% v/v acetic acid), (b) Amphotericin A dissolved in neutral methanol, and (c) mixture of solutions (a) and (b). The standard samples contained (a,c) 20 μg of Amphotericin B and (b,c) 11 μg of Amphotericin A at a concentration of 1. mg/ml. A Waters μC_{18} column was used with a methanol/dimethylformamide solvent system as described in the text. The absorption of effluent was monitored at 280 nm.

TABLE 7
Solvent Systems for Thin Layer Chromatography

<u>System</u>	<u>Solvent</u>	<u>R_f</u>	<u>Reference</u>
A	CHCl ₃ :CH ₃ OH:Borate Buffer (7:5:1) pH 8.3	0.60	47
B	N-butanol:C ₂ H ₅ OH:CH ₃ COOH:H ₂ O (50:15:15:20)	0.6	50
C	N-butanol:CH ₃ COOH:H ₂ O (3:1:1)	0.5	50
D	CH ₃ OH:Acetone:CH ₃ COOH (8:1:1)	0.45	48
E	CHCl ₃ :CH ₃ OH:20% NaOH (2:2:1)	0.4	50
F	Pyridine: ethylacetate: H ₂ O (25:16:7)	0.4	50
G	Butan-1-ol:pyridine:H ₂ O (3:2:1)	0.32	49
H	N-butanol (H ₂ O saturated)	0.2	50
I	C ₂ H ₅ OH: ammonia:dioxan-H ₂ O (8:1:1:1)	0.19	49
J	CH ₃ OH:propan-2-ol:CH ₃ COOH (90:10:1)	0.18	48
K	Butan-1-ol: ammonia:methanol:H ₂ O (20:1:2:4)	0.07	47

TABLE 8
Minimal Inhibitory Concentration (MIC)
of Amphotericin B

<i>Candida albicans</i>	1.9
<i>Candida tropicalis</i>	25.0
<i>Candida pseudotropicalis</i>	7.3
<i>Candida parakrusei</i>	1.1
<i>Cryptococcus neoformans</i>	0.2
<i>Epidermophyton floccosum</i>	0.2
<i>Fusarium bulbigenum</i>	14.7
<i>Microsporum canis</i>	7.3
<i>Microsporum audouini</i>	
<i>Rhodotorula glutinis</i>	0.9
<i>Rhodotorula mucilagenosa</i>	1.9
<i>Saccharomyces cerevisiae</i>	1.8
<i>Sporotrichum schenckii</i> (yeast phase)	0.07
<i>Trichophyton megnini</i>	0.9
<i>Trichophyton mentagrophytes</i>	2.4
<i>Trichophyton gallinae</i>	7.3
<i>Trichophyton rubrum</i>	7.3
<i>Trichophyton tonsurans</i>	4.9
<i>Monosporium apiospermum</i>	30.0

MIC was $> 40 \mu\text{g/ml}$ for:

<i>Aspergillus fumigatus</i>	<i>Microsporum gypseum</i>
<i>Candida parapsilosis</i>	<i>Nocardia asteroides</i>
<i>Cephalosporium recifei</i>	<i>Nocardia asteroides mexicana</i>
<i>Cladosporium carrionii</i>	<i>Nocardia brasiliensis</i>
<i>Cladosporium werneckii</i>	<i>Nocardia madurae</i>
<i>Fonsecaea pedrosoi</i>	<i>Philaophora verrucosa</i>
<i>Fonsecaea compactum</i>	<i>Sporotrichum schenckii</i>
<i>Geotrichum sp.</i>	(mycelial phase)

Note: From Reference 1; MIC ($\mu\text{g/ml}$) measured on second day after inoculation of agar medium.

Amphotericin A, and several other antibiotics in various electrolyte systems have been reported (57).

6. ISOLATION

In the original method of Vandeputte, et al., (1), *Streptomyces nodosus* (M 4575) whole broth is mixed with isopropanol (1:1) and adjusted to pH 10.5. The filtrate is neutralized, the alcohol evaporated, and the resulting powder (40-70% pure) washed with water and acetone, and vacuum dried. Slurrying with a 2% CaCl_2 methanol solution separates Amphotericin A (filtrate) and Amphotericin B (precipitate). The B fraction is slurried with acidic DMF, followed by dilution of the filtrate in methanol and precipitation with water while maintaining pH 5. The precipitate (75-80% pure) is again dissolved in acidic DMF, diluted with pure methanol, and precipitated with water. Amphotericin A (65-70%) results from adding water to the A filtrate, and drying the precipitate. (Methanolic CaCl_2 solubilization and water precipitation can be repeated to remove the remaining Amphotericin B.)

7. STABILITY

Dry Amphotericin B powder appears stable for long periods of time at room temperature (1,11). Isopropanol: H_2O (1:1) solutions are stable for days at pH 6-8, less stable at pH 4, 10 and decompose rapidly at pH 12 (1). The stability at 70°C (pH 7) is half that at 30°C (1). Solutions in phosphate-citrate buffer ($5 < \text{pH} < 7$) are apparently stable (58). In dextrose infusions at room temperature, Amphotericin B aggregates in the presence of NaCl (25% reduction of activity within 4 hours).

The activity of aqueous, clinically prepared dextrose solutions ($\text{pH} > 4$) did not decrease appreciably during an 8-hour exposure to 100-foot candles of ambient fluorescent light (59). After 3 days exposure to light in other experiments, biological (but not colorimetric) assays showed a 26% loss in activity (60).

Heating dry samples for 16 hours at 105°C results in only ~17% loss of potency. In contrast, 15 minutes at 158°C (above the chemical transition of Section 2.11) is sufficient to cause an ~21% loss of potency (21). Vibrator grinding of the sample at room temperature causes an ~30% loss of potency (average activity 688 mcg/min, rather than 986 mcg/min; Reference 61) as measured by the *Saccharomyces Cervisiae* assay of Reference 30.

8. ANTIMICROBIAL PROPERTIES AND ASSAYS

Minimal inhibitory concentrations (MIC) of Amphotericin B are given in Table 8 for several organisms (1). Stock

solutions were made in DMSO (4 mg/ml) and diluted in distilled water; the fungi were plated on agar (broth dilution assays give somewhat different results). The data of Table 8 are for the second day of observation.

Assay procedures utilizing Saccharomyces cerevisiae, Candida albicans, or Candida tropicalis are described in References (1,3,16). The Code of Federal Regulations (30) prescribes a microbiological agar diffusion assay suitable for pharmaceutical formulations using Saccharomyces cerevisiae (ATCC 9763). Additional biological assays can be found in Reference 3 and are summarized in Table 9.

The binding of Amphotericin B to S. cerevisiae has been investigated using fluorescence (62). Weak, reversible binding occurs even at 0°C and in the presence of metabolic inhibitors; it appears to affect only the outside of the membrane. In contrast, antimicrobial action involves the loss of essential cellular constituents as a result of strong, irreversible binding to the membrane. This strong binding, which can be blocked by cooling to 0°C or by metabolic inhibitors, apparently disrupts the deeper hydrophobic portions of the membrane. Enhanced fluorescence assays are reported to be linear in the range 0.1 - 10. μM (62).

Serum and urine can be assayed by agar diffusion for Amphotericin B activity with a sensitivity of about 0.01 mcg/ml (63). An equally sensitive turbidimetric microbiological assay (64) has been developed for use with small samples (e.g., 25 μl of serum or spinal fluid). These methods are summarized in Table 9. Feces levels can be determined by spectrophotometry of simple DMSO extracts, making use of a correction for the high baseline absorption (64).

9. AMPHOTERICIN A

Amphotericin A ($\text{C}_{46}\text{H}_{73}\text{NO}_{19}$, Reference 13) is isolated from *Streptomyces Nodosus*, along with Amphotericin B which it closely resembles (1). It is, however, a tetraene (like nystatin) and is thus readily distinguished from Amphotericin B by its ultraviolet absorption spectrum: 228, 280, 291, 304, 318 nm (1,18). Its specific rotation $[\alpha]_D^{24}$ (-9.9° in 0.1N methanolic HCl; +32° in "acidic" DMF) is also distinctive (1,3; but see Section 3.4). In contrast, infrared spectra (1,18,34) are highly similar, but not identical to Amphotericin B.

Amphotericin A is far more soluble in CH_3OH , DMF, water-saturated propanol or butanol, and CH_3COOH than Amphotericin B (1). Unlike Amphotericin B, it forms a water soluble sodium salt in methanolic -NaOH and a methanol soluble CaCl_2 complex; the latter property was used in its original

TABLE 9
Microbiological Assay Methods for
Amphotericin B

<u>Type of Sample</u>	<u>Method</u>	<u>Test Culture</u>	<u>Reference</u>
Formulated and unformulated products	Diffusion	<u>Saccharomyces cerevisiae</u> N.C.Y.C. 87	65
	Diffusion	<u>Saccharomyces cerevisiae</u> ATCC 9763	66
	Turbidimetric	<u>Candida tropicalis</u> ATCC 13803	64
Body Fluids	Diffusion	<u>Paecilomyces varioti</u> MSSC 5605 NIAID	63
	Turbidimetric (Micro scale)	<u>Candida tropicalis</u> ATCC 13803	64
Animal Feeds	Diffusion	<u>Saccharomyces cerevisiae</u> ATCC 9763	67

isolation (1). Amphotericin A can (presumably) be separated from Amphotericin B and nystatin by the thin-layer chromatographic methods of References 49 and 68 respectively. It can be reliably separated from Amphotericin B by high-pressure liquid chromatography (Section 5.3).

Amphotericin A is several times less active than Amphotericin B (59) and is usually encountered as a contaminant of the latter. Amphotericin A is considerably more sensitive to catalytic hydrolysis, and is thus less stable in aqueous isopropanol (1).

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BETAMETHASONE DIPROPIONATE

Michael G. Ferrante and Bruce C. Rudy

INDEX

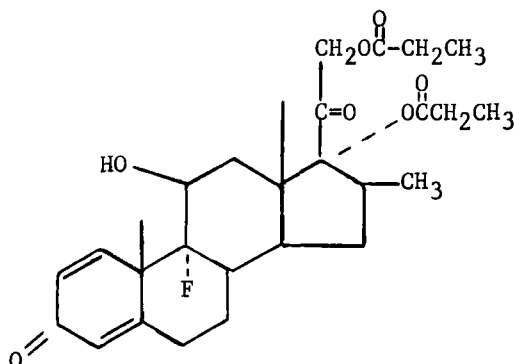
Analytical Profile - Betamethasone Dipropionate

1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance
2. Physical Properties
 - 2.1 Infrared Spectrum
 - 2.2 Nuclear Magnetic Resonance Spectrum
 - 2.3 Mass Spectrum
 - 2.4 Ultraviolet Spectrum
 - 2.5 Optical Rotation
 - 2.6 Melting Range
 - 2.7 Differential Scanning Calorimetry
 - 2.8 Thermogravimetric Analysis
 - 2.9 Solubility
 - 2.10 Xray Diffraction
3. Synthesis
4. Stability
5. Method of Analysis
 - 5.1 Elemental Analysis
 - 5.2 Thin Layer Chromatographic Analysis
 - 5.3 Liquid Chromatographic Analysis
 - 5.4 Direct Spectrophotometric Analysis
 - 5.5 Colorimetric Analysis
6. References

1. Description

1.1 Name, Formula, Molecular Weight

The chemical name for betamethasone dipropionate is 9 α -fluoro-11 β -hydroxy-16 β -methyl-17 α 21-dipropionyloxy-pregna-1,4-diene-3,20-dione.



$C_{28}H_{37}FO_7$

Molecular Weight 504.6

1.2 Appearance

Betamethasone dipropionate is a white to cream colored powder.

2. Physical Properties

2.1 Infrared Spectrum (IR)

The infrared spectrum of betamethasone dipropionate is presented in Figure 1. The spectrum was obtained as a mineral oil mull on a Perkin-Elmer Model 180 grating infrared spectrophotometer. The assignments for the characteristic bands in the infrared spectrum are listed in Table I.¹

Figure 1

INFRARED SPECTRUM OF BETAMETHASONE DIPROPIONATE

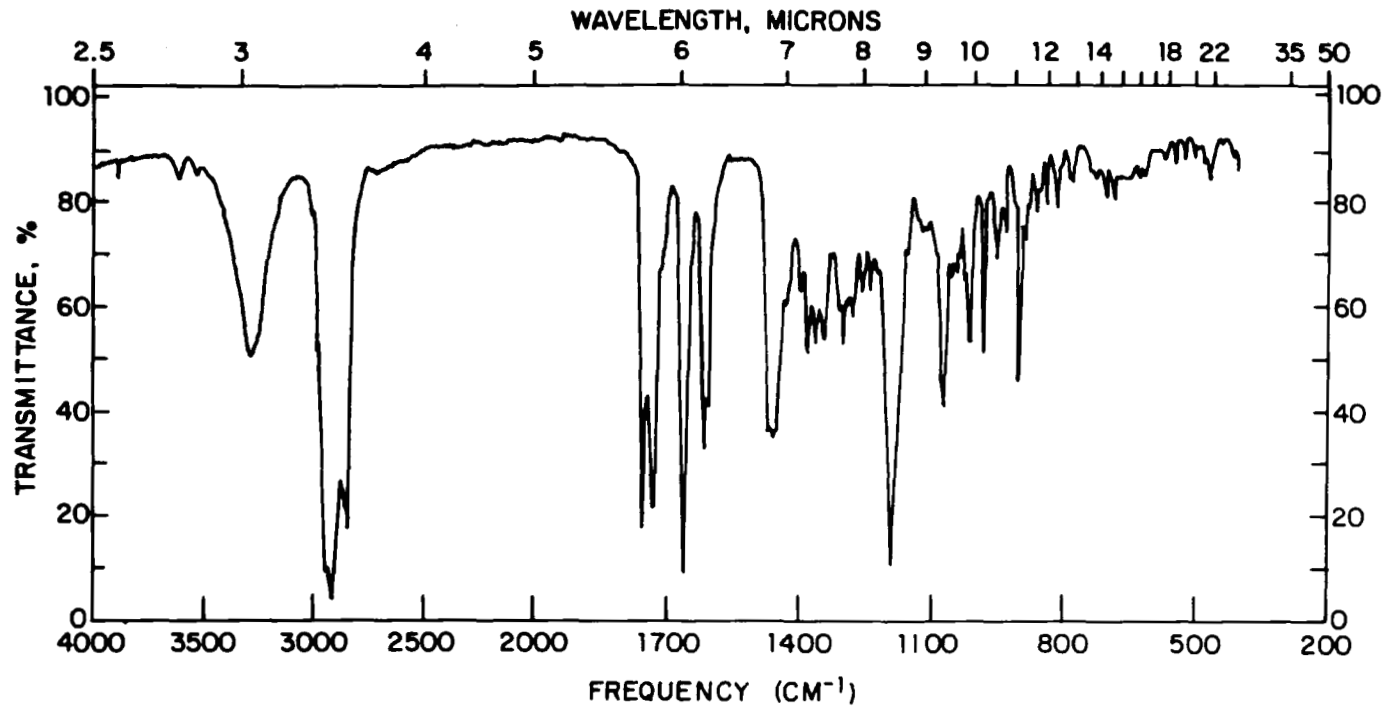


Table I

IR Assignments for Betamethasone Dipropionate

<u>Frequency (cm⁻¹)</u>	<u>Intensity*</u>	<u>Characteristic of</u>
3300	m	O-H stretch
3025, 3000	w	C-H stretch, $\Delta^{1,4}$
1755, 1728	s, d	C=O stretch, 17,21-dipropionate, 20-ketone
1660	s	C=O stretch, 3-ketone
1620, 1608	s, d	C=C stretch, $\Delta^{1,4}$ -diene
1189	s	C-O stretch, propionate ester
1068	m	C-O stretch, 11-hydroxyl

*s=strong, m=medium, w=weak, d=doublet

2.2 Nuclear Magnetic Resonance Spectrum (NMR)

The 100 MHz Fourier transform proton NMR spectra of betamethasone dipropionate, Figure 2, was obtained on a Varian XL-100-15 spectrometer at ambient temperature in CDCl₃ solvent with a concentration of 20 mg/ml. Chemical shifts are reported in ppm (δ) downfield from internal tetramethylsilane (TMS) in Table II.²

Table II

NMR Assignments for Betamethasone Dipropionate

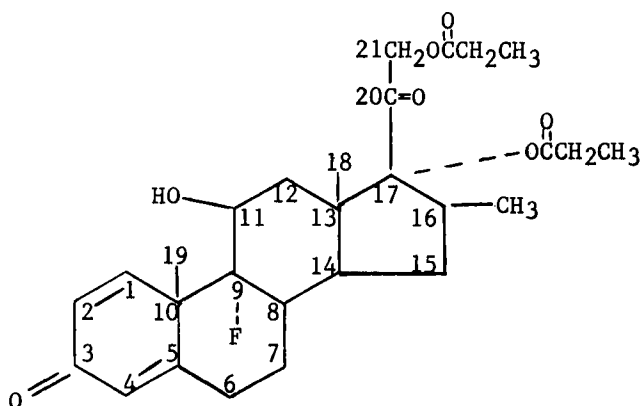
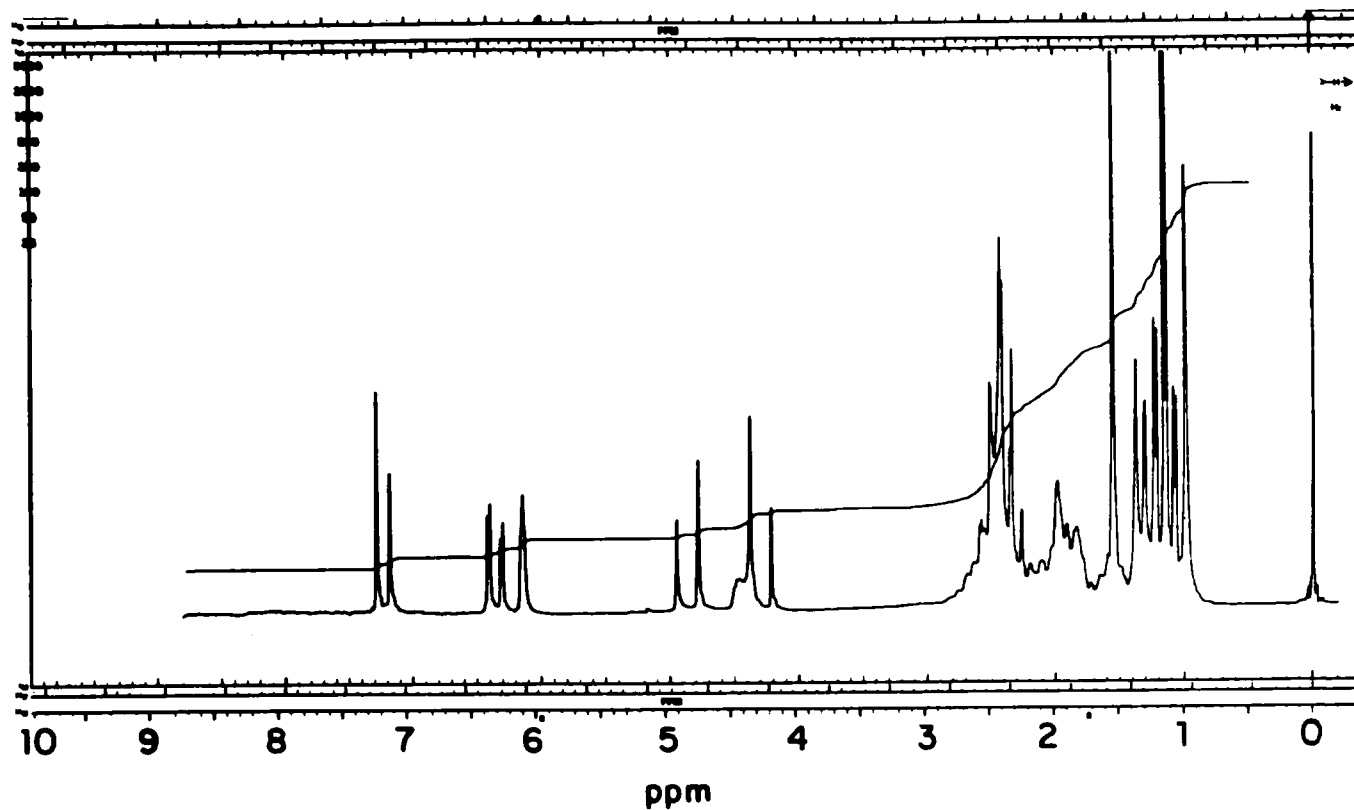


Figure 2
NMR SPECTRUM OF BETAMETHASONE DIPROPIONATE



<u>Proton</u>	<u>Chemical Shift (δ)</u>	<u>Multiplicity</u>
C13-CH ₃	0.92	Singlet
C16-CH ₃	1.27	Doublet
C10-CH ₃	1.52	Singlet
11 α -H	4.30	Multiplet
21-H	4.45	Doublet
21'-H	4.80	Doublet
*11 β -O-H	5.52	Doublet
C4-H	6.04	Broad singlet
C2-H	6.26	Doublet of doublets J ₁ , =10 Hz; J ₂ , =1.5 Hz
C1-H	7.30	Doublet
C17 and C21 Propionate methyls	1.05 and 1.09	Triplet
C17 and C21 Propionate methylenes	2.42	Quartet

*Chemical shift and coupling constant vary with concentration and temperature, but disappears when D₂O is added.

2.3 Mass Spectrum

The mass spectrum of betamethasone dipropionate was obtained at 70eV on a Varian MAT CH5 medium resolution single focusing (magnetic sector) instrument, interfaced with a Varian SS-100C data system, at a probe temperature of 170°C and a source temperature of 250°C. The data system utilized the output of the spectrometer to determine the masses, then compared their intensities to the base peak (100% intensity) and produced the bar graph in Figure 3.³

A listing of the prominent fragments and their resulting masses are given in Table III.

Figure 3
MASS SPECTRUM OF BETAMETHASONE DIPROPIONATE

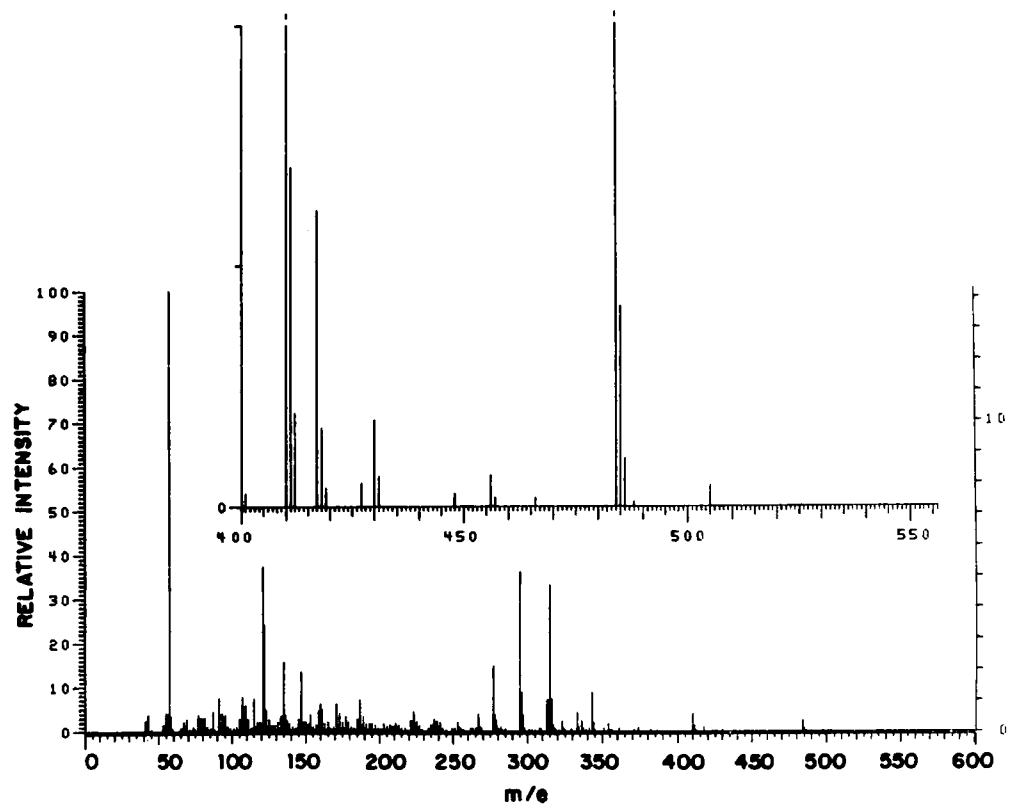


Table III

Mass Spectrum Assignments for Betamethasone Dipropionate

<u>Mass</u>	<u>Ion</u>	<u>Fragments Lost</u>
505	M+1	
484	M-20	HF
417	M-87	$\text{CH}_2\text{OC}(=\text{O})\text{CH}_2\text{CH}_3$
410	M-94	$\text{HF} + \text{CH}_3\text{CH}_2\text{COOH}$
343	M-161	$\text{CH}_2\text{OC}(=\text{O})\text{CH}_2\text{CH}_3 + \text{CH}_3\text{CH}_2\text{COOH}$
336	M-168	$\text{HF} + 2\text{CH}_3\text{CH}_2\text{COOH}$
333	M-171	$\text{COCH}_2\text{OC}(=\text{O})\text{CH}_2\text{CH}_3 + \text{C}_2\text{H}_4\text{CO}$
315	M-189	$\text{COCH}_2\text{OC}(=\text{O})\text{CH}_2\text{CH}_3 + \text{CH}_3\text{CH}_2\text{COOH}$
295	M-209	$\text{COCH}_2\text{OC}(=\text{O})\text{CH}_2\text{CH}_3 + \text{CH}_3\text{CH}_2\text{COOH} + \text{HF}$
277	M-227	$\text{COCH}_2\text{OC}(=\text{O})\text{CH}_2\text{CH}_3 + \text{CH}_3\text{CH}_2\text{COOH} + \text{H}_2\text{O}$
267	M-237	$\text{COCH}_2\text{OC}(=\text{O})\text{CH}_2\text{CH}_3 + \text{CH}_3\text{CH}_2\text{COOH} + \text{CO}$

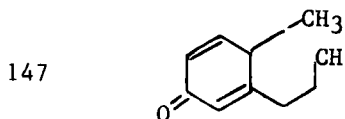
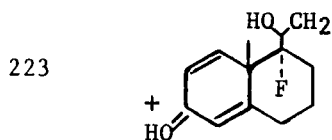
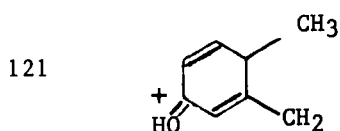
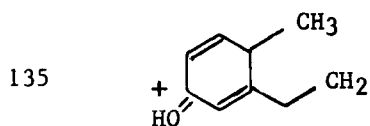


Table III (Continued)

Mass Spectrum Assignments for Betamethasone Dipropionate

<u>Mass</u>	<u>Ion</u>	<u>Loss</u>
-------------	------------	-------------

2.4 Ultraviolet Spectrum (UV)

When the ultraviolet spectrum of betamethasone dipropionate was scanned from 350 to 210 nm, a single maxima was observed at 238 nm ($\epsilon=1.57 \times 10^4$). The spectrum in Figure 4 was obtained from a solution of 3.056 mg of betamethasone dipropionate in 100.0 ml of methanol.

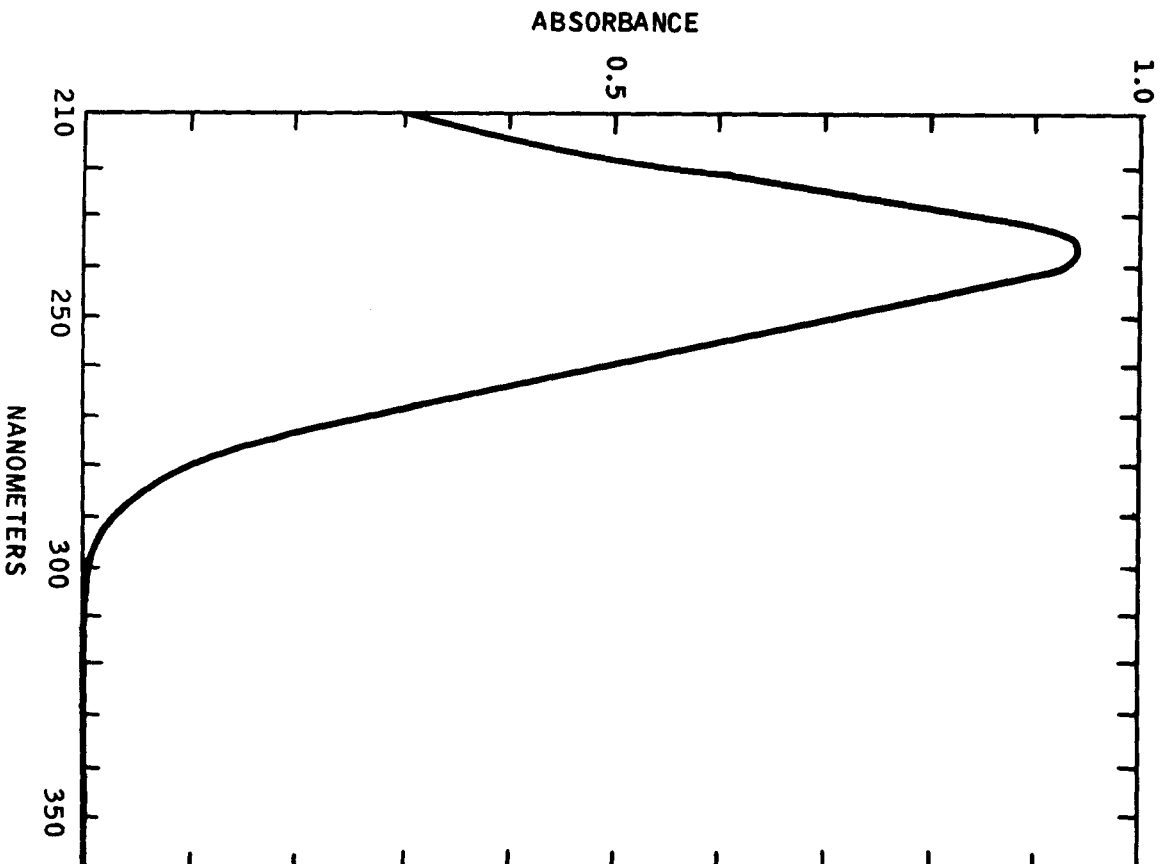
2.5 Optical Rotation

Betamethasone dipropionate exhibited the following specific rotations:⁴

$$\left[\alpha \right]_{26}^{\text{Dioxane}} = + 65.7^{\circ}$$

$$\left[\alpha \right]_{27}^{\text{Methanol}} = + 89.4^{\circ}$$

Figure 4
ULTRAVIOLET SPECTRUM OF
BETAMETHASONE DIPROPIONATE



2.6 Melting Range

Betamethasone dipropionate melts in a 3° range between 170° and 179°C with decomposition, when the USP XVIII class Ia procedure is used.⁵

2.7 Differential Scanning Calorimetry (DSC)

The DSC curve for betamethasone dipropionate obtained at a scan rate of $10^{\circ}\text{C}/\text{min.}$ is shown in Figure 5. The curve was recorded with a DuPont 900 Differential Thermal Analyzer under an atmosphere of nitrogen flowing at 200 cc/min. A single endotherm was observed, the extrapolated onset of melting occurred at 175°C .⁶

2.8 Thermogravimetric Analysis (TGA)

The TGA curve for standard betamethasone dipropionate exhibited no weight loss on a scan from 27° to 175°C at $10^{\circ}\text{C}/\text{min.}$ ⁷

2.9 Solubility

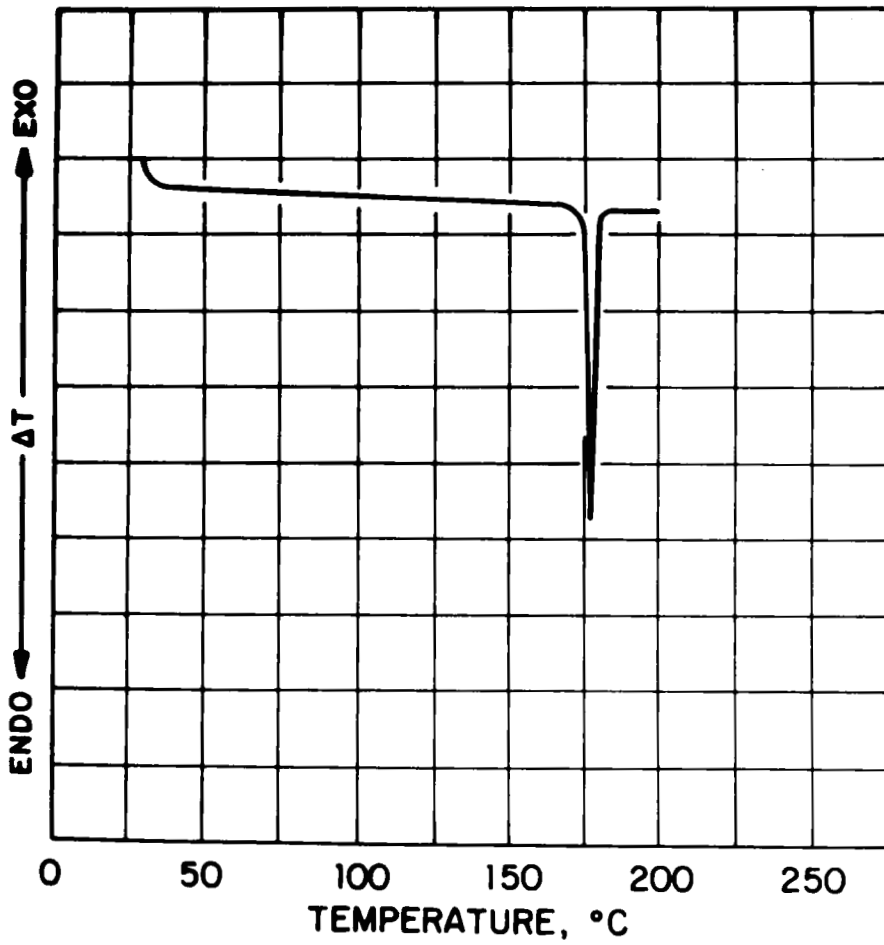
The solubility data for betamethasone dipropionate is listed in Table IV.⁸

Table IV

Betamethasone Dipropionate Solubility Measurements

<u>Solvent</u>	<u>Solubility mg/ml, 25°C</u>
Acetone	>100
Benzene	30
Chloroform	>100
Dimethylformamide	>100
Dimethylsulfoxide	>100
Ethanol (USP)	45
Ethanol (USP) 85% - Water 15% (v/v)	30
Ether	5
Ethyl Acetate	70
Methanol	55
Mineral Oil	<0.05
Petroleum Ether	<0.03
Polyethylene Glycol 400	26
Propylene Glycol	7
Water	<0.04

Figure 5
DSC OF BETAMETHASONE DIPROPIONATE



2.10 Xray Diffraction

The xray diffraction spectrum of betamethasone dipropionate is presented in Table V.⁹ The data were collected on a Philips APD-3500 utilizing Cu K α radiation (1.5418 \AA).

Table V

Xray Data for Betamethasone Dipropionate

<u>d(\AA)</u>	<u>I/I'</u>	<u>d(\AA)</u>	<u>I/I'</u>
41.72	50	5.290	77
39.112	52	4.862	55
38.024	52	4.622	12
36.392	51	4.602	13
34.864	52	4.572	14
33.944	50	4.520	12
32.880	49	4.507	12
30.234	46	4.465	18
28.685	43	4.421	24
25.022	31	4.405	24
23.909	26	3.948	28
9.700	22	3.893	40
9.291	54	3.845	26
9.664	40	3.596	20
8.046	45	3.370	37
7.047	15	3.359	37
6.082	100	3.030	24
5.703	71	3.020	24

3. Synthesis

Betamethasone dipropionate is prepared by the following synthesis. Betamethasone is reacted with ethyl ortho-propionate and toluene-p-sulphonic acid to yield betamethasone 17,21-ethylorthopropionate.¹⁰ This compound is then reacted with acetic acid to yield betamethasone 17-propionate.¹¹ This intermediate product is then treated with propionyl chloride at 0°C, diluted with water and acidified with dilute hydrochloric acid. This yields the crude diester which when recrystallized yields the final pure form of betamethasone dipropionate.¹²

4. Stability

Betamethasone dipropionate has a high stability in aqueous suspensions as compared to other corticosteroids. This may be attributed to its diester structure and correspondingly low solubility in water. The compound is most stable at pH 4, with any hydrolyzation resulting in the formation of betamethasone alcohol.¹³ At the extremes of pH, large amounts of more polar products were observed which although not identified, can be assumed to be further breakdown products of the dihydroxy acetone side chain.¹⁴

Betamethasone dipropionate is stable towards air oxidation in the solid state. Heating of the compound at 75 C for 6 months in the presence of air shows no change in color or in the thin layer chromatogram.¹⁵

Over long periods of exposure to fluorescent light, there is minor degradation of the drug.¹⁶ It should also be expected that solutions of betamethasone dipropionate are subject to photolytic degradation since photolytic degradation of the A-ring of steroidal 1,4-diene-3-ones has been reported in literature.¹⁷

5. Method of Analysis

5.1 Elemental Analysis

The results of elemental analysis on a sample of standard betamethasone dipropionate are presented below.¹⁸

<u>Element</u>	<u>Theory</u>	<u>Found</u>
C	66.65	66.54
H	7.40	7.18
F	3.77	3.65

5.2 Thin Layer Chromatographic Analysis (TLC)

A TLC system which is used in the analysis of betamethasone dipropionate is as follows. The sample is applied to a silica gel GF plate and subjected to ascending chromatography using chloroform:acetone (7:1) as the developing solvent.

After the solvent is allowed to ascend 15 cm, the plate is air dried. This plate is then viewed under a shortwave ultraviolet light to identify and locate the betamethasone dipropionate band. The approximate Rf value is 0.5.¹⁹

5.3 Liquid Chromatographic Analysis

A high pressure liquid chromatography system for the separation and detection of betamethasone dipropionate was developed using the parameters listed below in Table VI.²⁰

Table VI

Column: Permaphase ODS* (DuPont) packed in a 1m x 2mm (i.d.) stainless steel column.
Detector: Ultraviolet detector at 254 nm.
Mobile Phase: Acetonitrile:water (1:3)
(degassed for 5 minutes using vacuum)
Pressure: 600 psi, adjustable
Flow Rate: 0.5 ml/min
Quantity Injected: 0.14 mg
Retention Times (minutes): Betamethasone monopropionates 5
Betamethasone dipropionate 7

*ODS - Octadecylsilane

5.4 Direct Spectrophotometric Analysis

Direct UV absorbances may be carried out on betamethasone dipropionate. A solution of betamethasone dipropionate is prepared containing approximately 0.02 mg/ml in methanol. The absorption spectrum of this solution is then recorded between 350 and 220 nm and compared to a similar solution of the standard.²¹

5.5 Colorimetric Analysis

The colorimetric analysis for betamethasone dipropionate involves utilization of the Mader-Buck reaction.²² A solution of betamethasone dipropionate is prepared containing approximately 0.016 mg/ml in ethanol (USP). To 20.0 ml of this solution is added 2.0 ml of blue tetrazolium solution (125 mg of blue tetrazolium reagent in 25 ml of USP ethanol) and 2.0 ml of tetramethylammonium hydroxide solution (10 ml of tetramethylammonium hydroxide, 10%, diluted to 100 ml with USP ethanol). This solution is then heated at 45°C in a water bath for 45 minutes. After heating, 1.0 ml of glacial acetic acid is added and the solution is allowed to cool. The absorption spectrum of this violet colored solution is read between 600 and 450 nm (λ_{max} = 525 nm).²³

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CLONAZEPAM

Walter C. Winslow

Contents

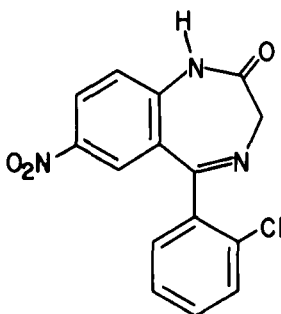
Analytical Profile - Clonazepam

1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor
2. Physical Properties
 - 2.1 Infrared Spectrum
 - 2.2 Nuclear Magnetic Resonance Spectrum
 - 2.3 Ultraviolet Spectrum
 - 2.4 Mass Spectrum
 - 2.5 Optical Rotation
 - 2.6 Melting Range
 - 2.7 Differential Scanning Calorimetry
 - 2.8 Thermogravimetric Analysis
 - 2.9 Solubility
 - 2.10 Crystal Properties
 - 2.11 Dissociation Constant
3. Synthesis
4. Stability Degradation
5. Drug Metabolism and Pharmacokinetics
6. Toxicology
7. Methods of Analysis
 - 7.1 Elemental Analysis
 - 7.2 Phase Solubility Analysis
 - 7.3 Chromatographic Analysis
 - 7.4 Electron Capture Gas Liquid Chromatography
 - 7.5 Spectrophotometric Analysis
 - 7.6 Polarographic Analysis
 - 7.7 Titrimetric Analysis
8. Acknowledgements
9. References

1. Description

1.1 Name, Formula, Molecular Weight

Clonazepam is (5-[2-chlorophenyl]-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one)



CLONAZEPAM

C₁₅H₁₀Cl N₃O₃

M.W. 315.7

1.2 Appearance, Color, Odor

Light yellow, crystalline powder which is practically odorless.

2. Physical Properties

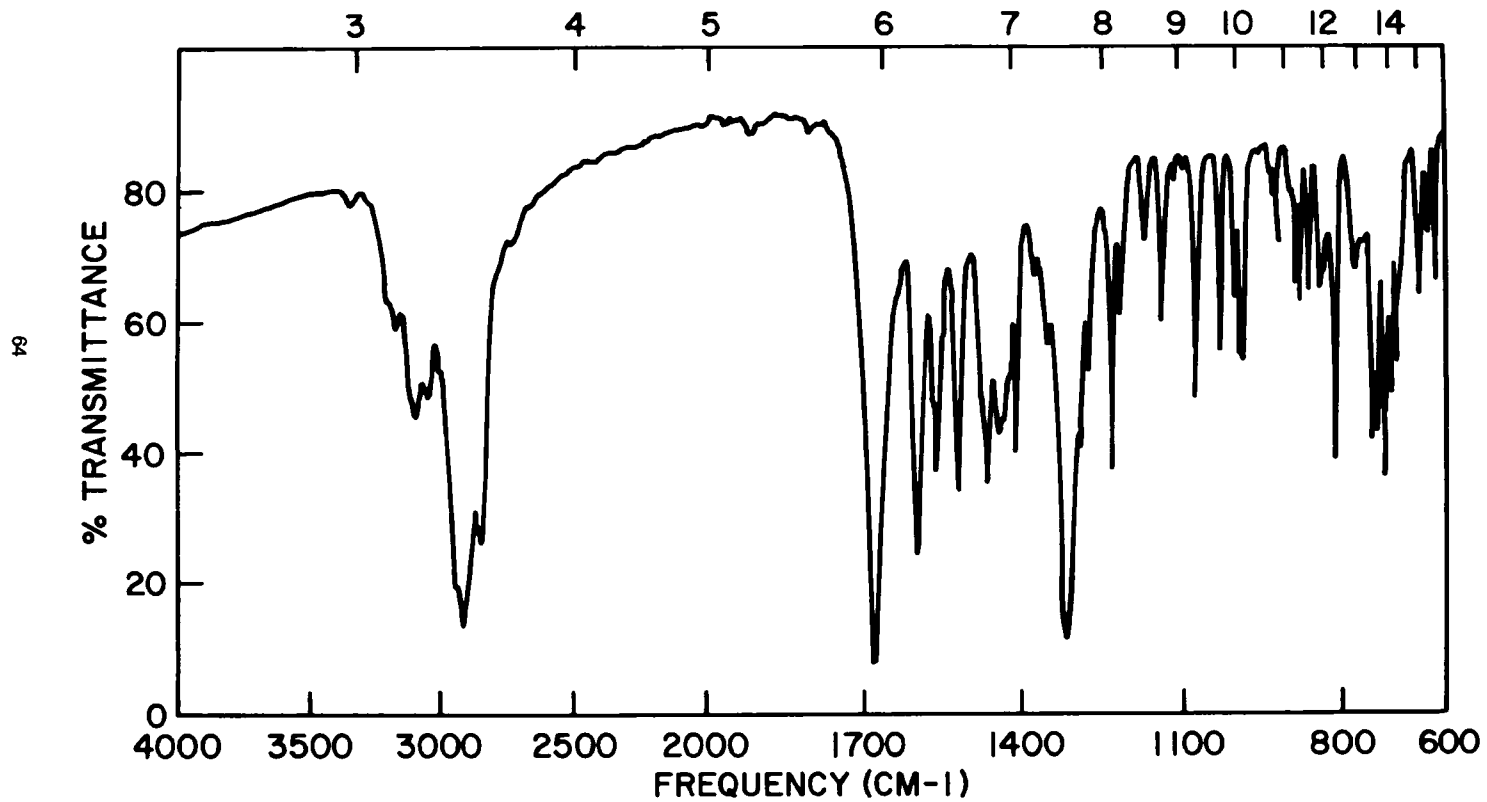
2.1 Infrared Spectrum

The infrared spectrum of a mineral oil suspension of reference standard clonazepam is presented in Figure 1.[¹] The spectral assignments are listed in Table 1.

Table 1

1. NH stretching: 3250-3100 CM⁻¹
2. Aromatic CH stretching: 3076, 3056 CM⁻¹
3. Carbonyl stretching: 1696 CM⁻¹
4. Aromatic Ring: 1615, 1582 CM⁻¹
5. Asymmetric NO₂ stretching: 1540 CM⁻¹
6. Symmetric NO₂ stretching: 1339 CM⁻¹
7. Aromatic CH out-of-plane bending:
 - 4 adjacent free H's: 750 CM⁻¹
 - 2 adjacent free H's: 844 CM⁻¹

FIGURE 1
IR Spectrum of Clonazepam
WAVELENGTH (MICRONS)



2.2 Nuclear Magnetic Resonance Spectrum (NMR)

The NMR spectrum of clonazepam is shown in Figure 2. The spectrum was determined on a JEOL C-60 HL spectrometer at ambient temperature (ca 25°C). The sample was dissolved in DMF-d₇ containing TMS as an internal reference. The spectral assignments are listed in Table 2.[²]

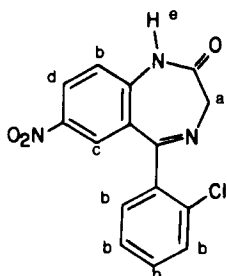


Table 2

<u>Proton</u>	<u>Chemical Shift δ (ppm)</u>	<u>Multiplicity</u>	<u>Coupling Const.J (Hz)</u>
a	4.48	Singlet	---
b	7.55-7.85	Multiplet	---
c	7.93	Doublet	2.5
d	8.50	Doublet (2 Sets)	2.5 (<i>meta</i> coupling)
e	11.30	Broad Singlet	8.6 (<i>ortho</i> coupling)

2.3 Ultraviolet Spectrum

The UV spectrum of clonazepam (1 mg of clonazepam in 100 ml of 7.5% methanol in isopropanol) in the region of 230 to 400 nm exhibits maxima at 248 nm ($\epsilon = 1.45 \times 10^4$) and 310 nm ($\epsilon = 1.16 \times 10^4$). Minima are observed at 239 and 279 nm. The spectrum is shown in Figure 3.[³]

FIGURE 2
NMR Spectrum of Clonazepam

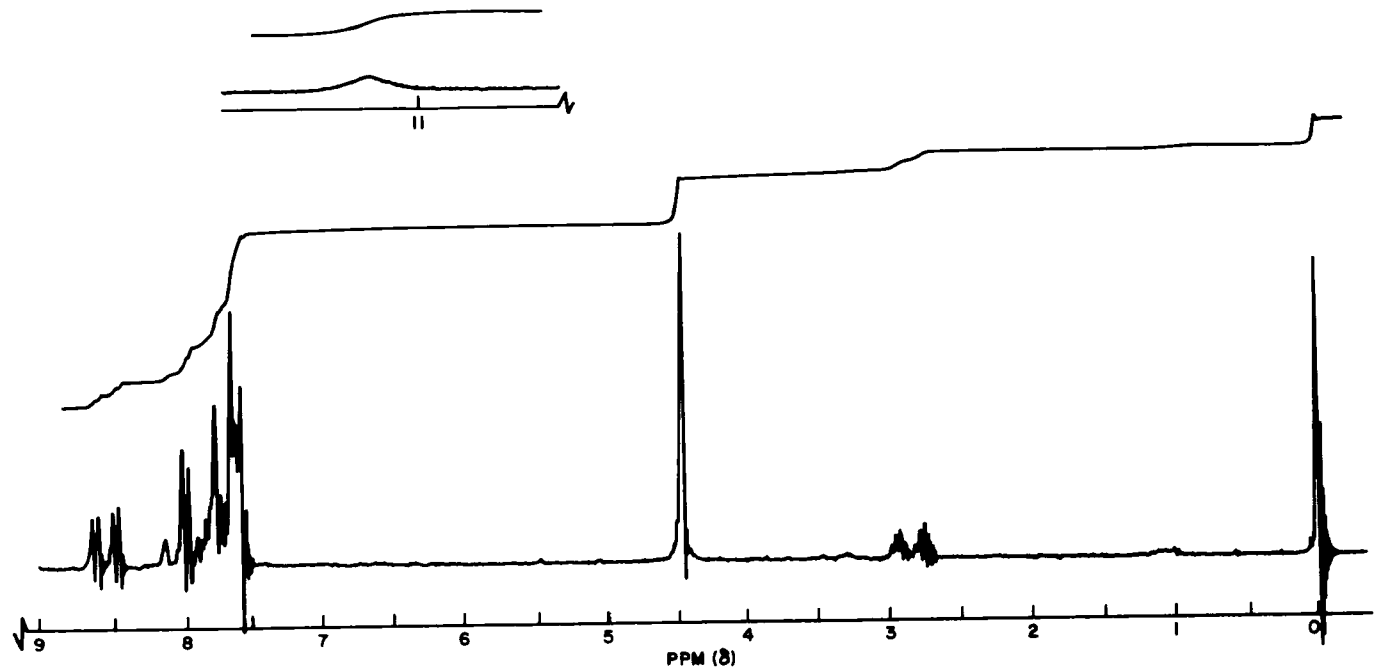
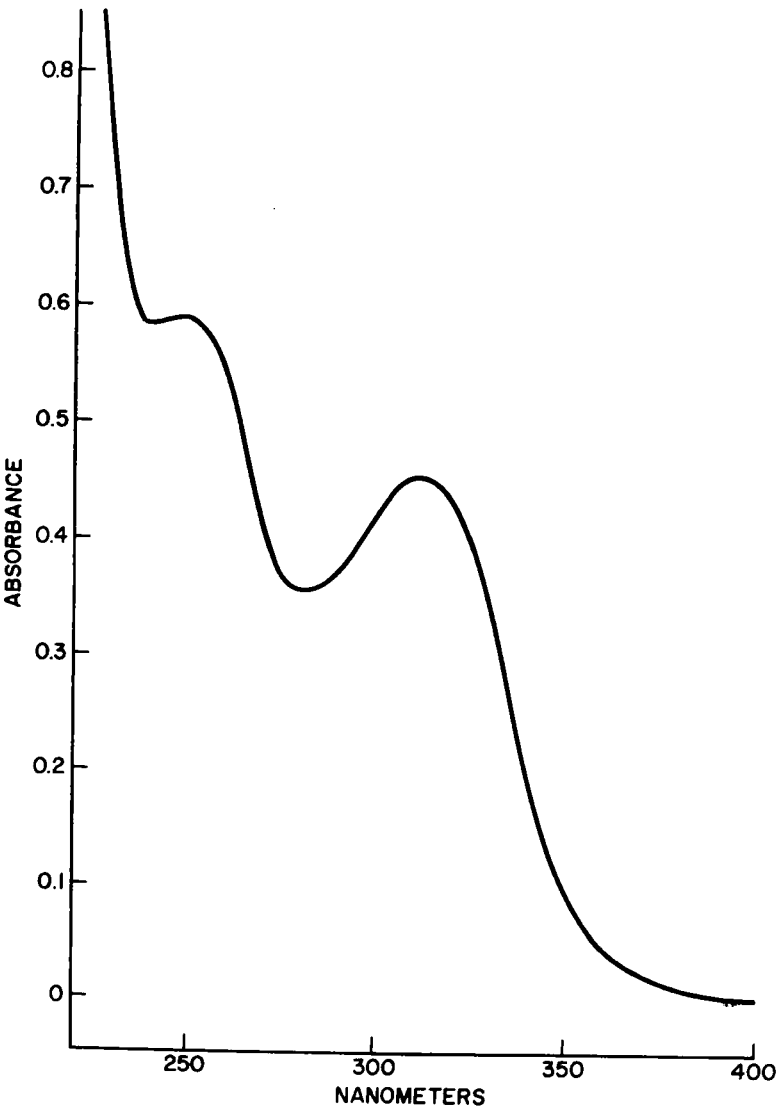


FIGURE 3
UV Scan of Clonazepam



2.4 Mass Spectrum

The low resolution mass spectrum of clonazepam is shown in Figure 4.^[4] The spectrum was run on a Varian CH 5 spectrometer interfaced with a Varian data-handling system. The computer calculates ion masses and compares their peak intensities to the base peak. This information is then automatically plotted as a series of lines whose heights are proportional to the peak intensities. The largest mass was observed at $m/e = 315$. The other characteristic peaks observed were:

<u>Mass (m/e)</u>	<u>Species</u>
315	M+
314	M+ - H
298	M+ - OH
286	M+ - CHO
280	M+ - Cl
268	314 - NO ₂
252	280 - CO, 280 - CH ₂ N
240	268-CO
234	280-NO
205	240-Cl

A high resolution scan confirmed the results of the low resolution spectrum.^[4] The elemental composition for the characteristic masses determined in the high resolution scan are shown in Table 3.

Table 3

<u>Mass Observed</u>	<u>Mass Calculated</u>	<u>C</u>	<u>H</u>	<u>N</u>	<u>O</u>	<u>Cl</u>
151.0541	151.0548	12	7	0	0	0
177.0596	177.0578	13	7	1	0	0
205.0763	205.0767	14	9	2	0	0
213.0354	213.0327	14	3	3	0	0
234.0797	234.0794	15	10	2	1	0
240.0437	240.0455	14	9	2	0	1
252.0531	252.0536	14	8	2	3	0
252.0760	252.0773	14	10	3	2	0
268.0433	268.0404	15	9	2	1	1
280.0701	280.0723	15	10	3	3	0
286.0370	286.0383	14	9	3	2	1
287.0308	287.0349	15	10	1	3	1
298.0362	298.0381	15	9	3	2	1
314.0294	314.0333	15	9	3	3	1
315.0364	315.0411	15	10	3	3	1

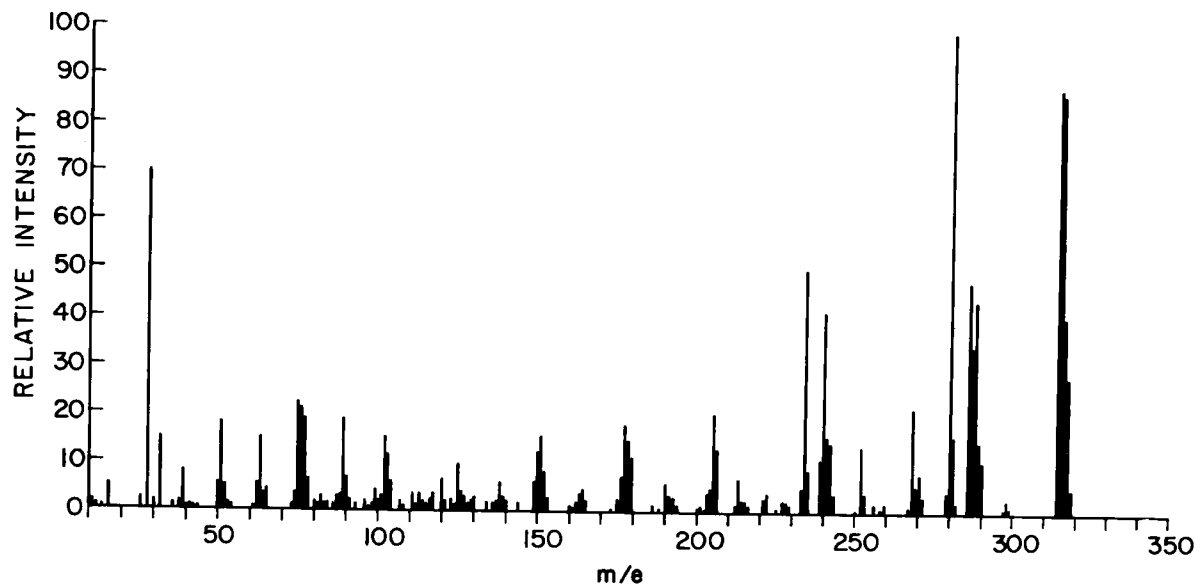


FIGURE 4
Mass Spectrum of Clonazepam

2.5 Optical Rotation

Clonazepam exhibits no optical activity.

2.6 Melting Range

Clonazepam melts between 237°C and 240°C when tested according to the USP XIX Class I procedure.[⁵]

2.7 Differential Scanning Calorimetry

The DSC thermogram of clonazepam at a heating rate of 10°C/minute is shown in Figure 5. A single endothermic transition, corresponding to the melting of the compound, is observed from 238.6°C to 240.2°C.[⁶]

2.8 Thermogravimetric Analysis

The TGA of clonazepam exhibited a single S shaped weight loss as a function of temperature. The loss started at ca. 195°C., reached 15% at 285°C. and then leveled off at 355°C. at which point 34% of the sample weight had been lost. Gradual weight loss continued until 500°C. (upper limit of instrument).[⁶]

2.9 Solubility

Approximate solubilities in various solvents, as determined gravimetrically from solutions equilibrated for 3 hours at 25°C, are given in Table 4.

Table 4

<u>Solvent</u>	<u>Solubility mg/ml</u>
Water	<0.1
95% Ethanol	5.6
Absolute Ethanol	4.7
Methanol	8.6
Isopropanol	2.3
Chloroform	15
Ethyl Ether	0.7
Benzene	0.5
Acetone	31
Ethyl Acetate	10
Propylene Glycol	5.2

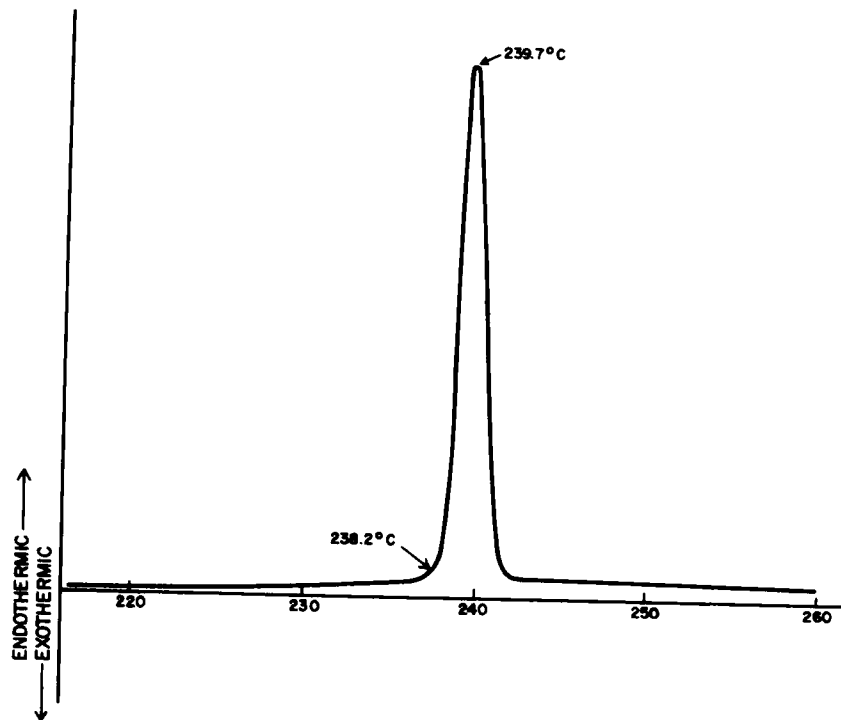


FIGURE 5
DSC Scan of Clonazepam

2.10 Crystal Properties

The X-Ray powder diffraction pattern of clonazepam is presented below.^[7]

Instrument Conditions

Instrument	GE Model XRD-6 Generator
Camera	Guinier-DeWolff II, with Pt-Rh Sample Screen
X-Ray Target	Chromium ($\text{CrK}_\alpha = 2.2909\text{\AA}$)
Focus	Line
Voltage	50 KV
Current	12.5 mA
Atmosphere	Helium
Exposure Time	2 Hrs.
Film	Ilford X-Ray Film Industrial G

<u>2θ</u>	<u>d(\AA)*</u>	<u>I/I_o**</u>
17.89	7.37	0.54
22.30	5.92	1.00
22.58	5.85	0.57
23.33	5.67	0.32
26.30	5.03	0.11
25.02	5.29	0.10
27.49	4.82	0.29
27.93	4.75	0.29
30.21	4.40	0.47
30.84	4.31	0.49
33.59	3.96	0.19
34.39	3.88	0.54
34.72	3.84	0.60
36.16	3.69	0.38
36.78	3.63	0.76
38.91	3.44	0.15
39.47	3.39	0.56
41.50	3.23	0.27
42.04	3.19	0.16

*d (interplanar distance) = $n\lambda/(2 \sin\theta)$

**I/I_o = relative intensity based on a maximum of 1.00

2.11 Dissociation Constant

The pKa values for clonazepam have been determined spectrophotometrically to be 1.5, corresponding to deprotonation of the nitrogen in the 4 position and 10.5 for the nitrogen in the 1 position.[⁸]

3. Synthesis

Clonazepam may be prepared by the reaction scheme shown in Figure 6.[⁹] o-Chlorobenzoyl chloride is reacted with p-nitroaniline in a modified Friedel-Crafts reaction to yield 2-amino-5-nitro-2'-chlorobenzophenone. The amino-ketone is then condensed with bromoacetyl bromide to form 2-bromoacetamido-5-nitro-2'-chlorobenzophenone. This compound is isolated and converted to the corresponding acetamido compound by reacting it in solution with ammonia. The ammonium bromide by-product is separated and the solvent removed. The residue is taken up in 5N anhydrous hydrogen chloride in methanol to form the hydrochloride salt which is then taken up in boiling ethanol. Pyridine is added which catalyzes ring closure to clonazepam.[¹⁰]

4. Stability Degradation

Degradation of clonazepam occurs principally via hydrolysis. Decomposition by this route is illustrated in Figure 7. The major breakdown products are 2-amino-2'-chloro-5-nitrobenzophenone (I) and 3-amino-4-(2-chlorophenyl)-6-nitrocarbostyryl (III).[^{11,12}] The latter is presumably formed via the aminoacetamido intermediate(II). Formation of the benzophenone results in a reduction in the absorptivity at 310 nm when measured in isopropanol, while formation of the carbostyryl leads to an increase in the absorptivity.[¹²]

5. Drug Metabolism and Pharmacokinetics

Clonazepam is an antiepileptic drug useful in the treatment of minor motor seizures which probably acts by potentiating inhibitory mechanisms in the subcortical brain structure responsible for the propagation of seizure activity.

Clonazepam, even in µg doses, protected mice from pentetrazole induced convulsions, and elevated the threshold for electroshock seizures in mice and cats. At very low doses clonazepam suppressed amygdalohippocampal evoked potentials in the cat and elevated the threshold for the

FIGURE 6
Synthesis of Clonazepam

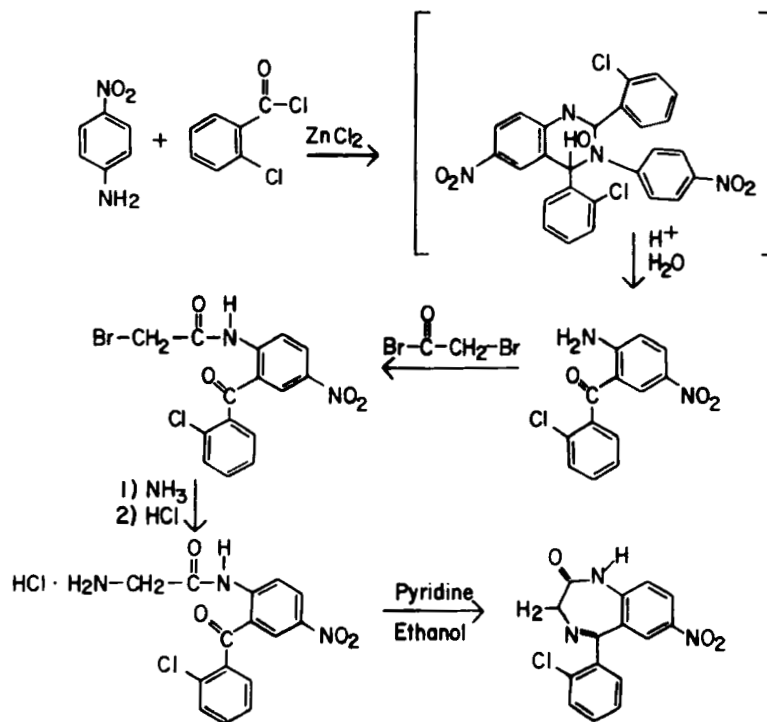
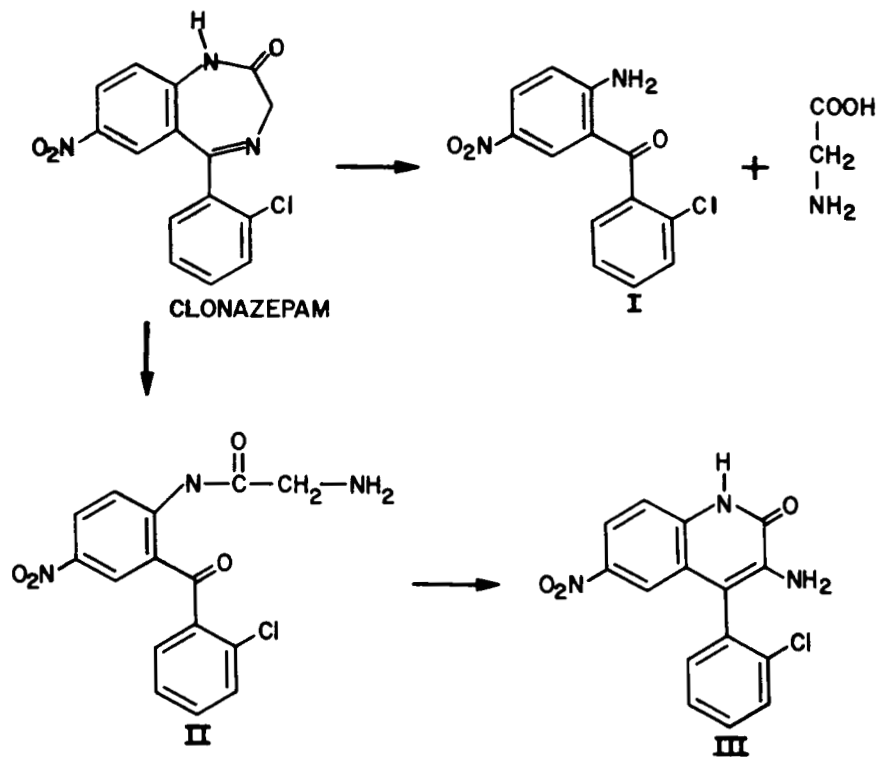


FIGURE 7
Decomposition of Clonazepam via Hydrolysis



generation of thalamic, but not cortical, after-discharges. On the spinal level, clonazepam depressed various motor reflex pathways and potentiated presynaptic inhibition as measured by the dorsal root potential.^[13]

The principle pathways of biotransformation were shown by Eschenhoff^[14] (Figure 8) to be reduction of the nitro group to an amine, subsequent acetylation of the amine and oxidative hydroxylation at C₃ which results in the elimination of these products as their glucuronides and/or sulfate conjugates. The half-life of the parent compound varies from 18 to 50 hours in humans and the major route of excretion is in the urine.^[15,16]

The two most prevalent metabolites of clonazepam have been found to be amino clonazepam and acetylamino clonazepam. Analytical procedures for detecting these compounds in body fluids, including differential pulse polarography^[16] and electron capture glc ^[16,17,18], have been reported.

6. Toxicology

The chronic tolerance of clonazepam in laboratory animals is excellent. The LD₅₀ for rats and mice: >4000 mg/kg by oral or i.p. administration and no fetotoxic effects were observed.^[13]

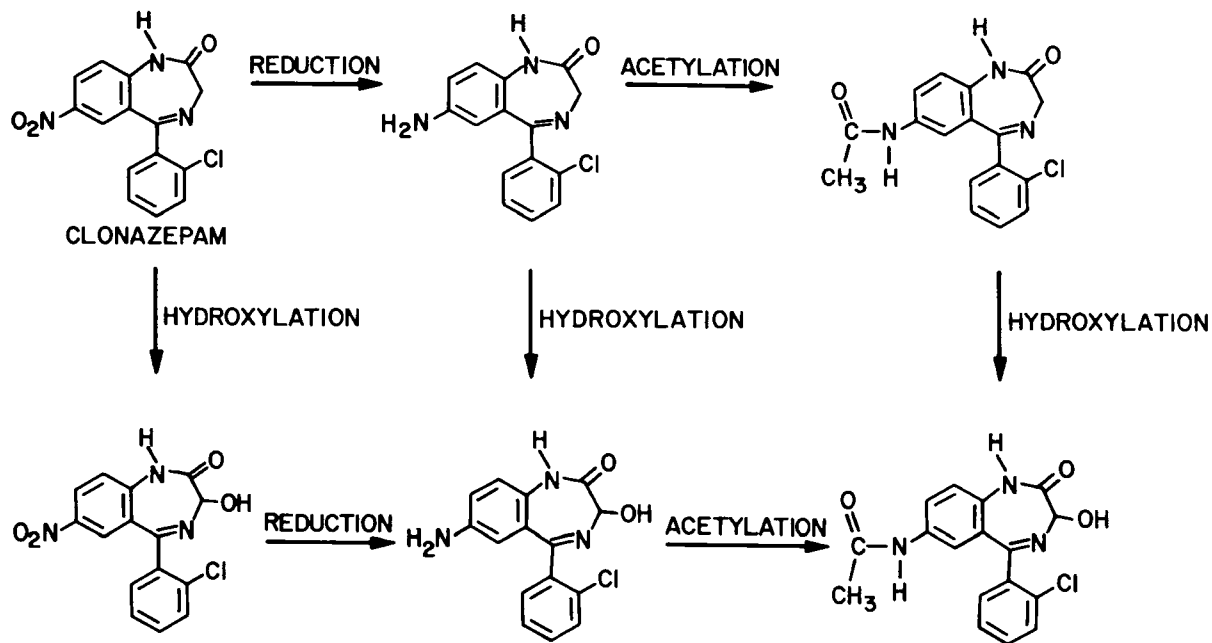
7. Methods of Analysis

7.1 Elemental Analysis

The elemental analysis of a sample of reference standard clonazepam is presented in Table 5.^[19]

<u>Table 5</u>		
<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
C	57.37	57.07
H	3.17	3.19
Cl	11.28	11.23
N	13.43	13.31
O	14.75	15.20 (by difference)

FIGURE 8
Metabolism of Clonazepam in Humans



7.2 Phase Solubility Analysis

Phase Solubility Analysis is carried out using methanol as a solvent. A typical example, listing the experimental conditions, is shown in Figure 9.

7.3 Chromatographic Analysis

Thin Layer Chromatography

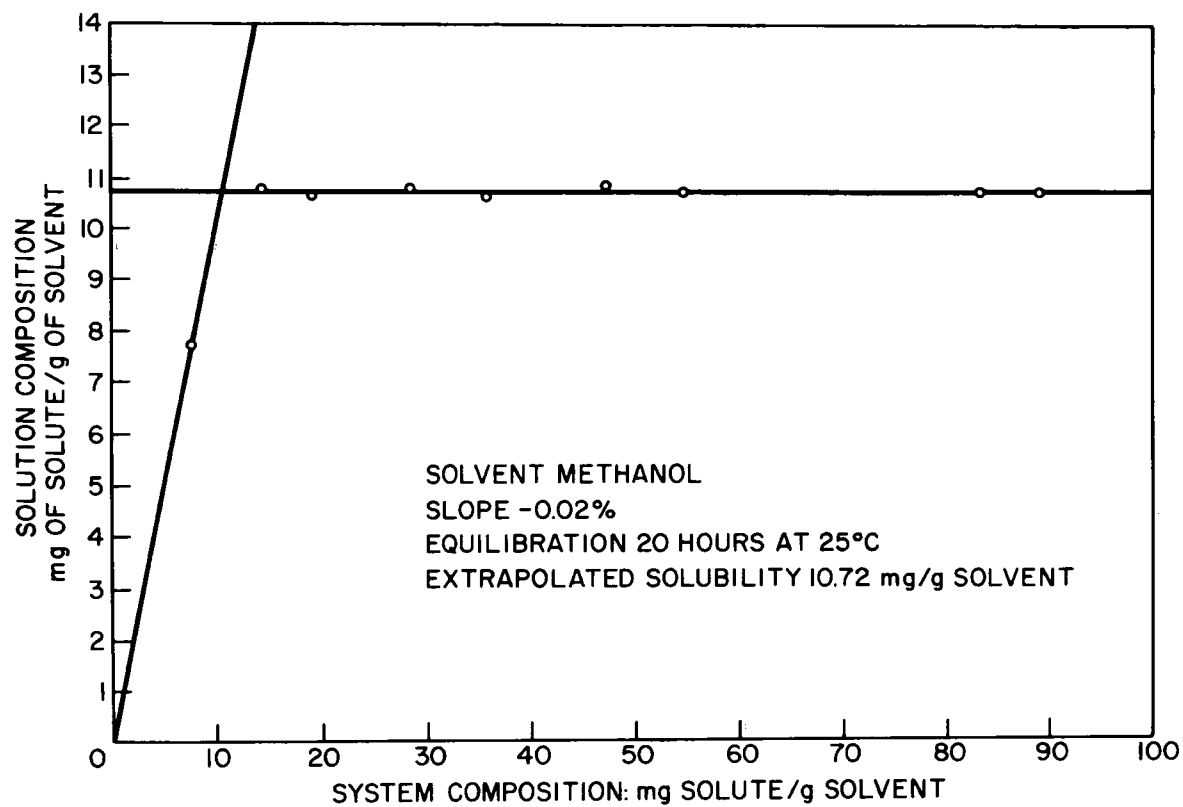
The following TLC systems are useful for identification and evaluation of clonazepam. System I^[20, 21] is a mixture of acetone:heptane 60:40 v/v. System II^[21] is ethyl acetate:carbon tetrachloride 50:50. In both systems, 20 μ l of sample solution, containing 0.5 mg of clonazepam in acetone, is applied to a silica gel GF plate and subjected to ascending chromatography. After development for about 15 cm the plates are removed and air dried. Detection is by examination of the plates under shortwave ultraviolet light. The plates are subsequently sprayed with 10% sulfuric acid and heated at 105°C for 15 minutes followed by diazotization and reaction with Bratton-Marshall reagent. The limit of detection for all species listed is at the 0.5 μ g level (0.1%). Approximate R_f values for clonazepam and related compounds are given below.

	<u>System I</u>	<u>System II</u>
<u>Species</u>	<u>R_f</u>	<u>R_f</u>
Clonazepam	.46	.43
Bromacetamido Impurity	.56	
Aminoacetamido Impurity	.64	
Carbostyрил		.60
Benzophenone		.90

7.4 Electron Capture Gas Liquid Chromatography

Methods for the determination of clonazepam in blood and urine have been reported which measure clonazepam directly,^[17] as its benzophenone^[16] and as its N-1-methyl derivative.^[18] Each of these methods is reported to have a sensitivity of approximately 1 ng/ml.^[22]

FIGURE 9
Phase Solubility Analysis of Clonazepam



7.5 Spectrophotometric Analysis

Spectrophotometric analysis of clonazepam may be carried out directly utilizing the UV maximum at 310 nm in isopropanol,^[12] however, as hydrolysis products of clonazepam may affect the absorptivity at this wavelength (see stability section), the absence of these species at appreciable levels should be confirmed by TLC.

7.6 Polarographic Assay

Clonazepam exhibits a dual reduction wave which may be attributed to the reduction of the 4,5-azomethine and nitro groups. Senkowski^[23] et al. showed that the polarographic reduction of these groups for various 1,4-benzodiazepines in 0.1N HCl in 20% methanol are sufficiently separated for quantitative work based on the reduction of the azomethine group at about - 0.6V vs. SCE. Linearity was obtained between sample concentration and the diffusion current. The polarographic assay of clonazepam has been performed in aqueous systems by De Silva et al.,^[16] with a sensitivity of 0.5 - 0.75 µg/ml.

7.7 Titrimetric Analysis

Clonazepam is assayed by dissolving the sample in acetic anhydride and titrating with 0.1N perchloric acid (HClO₄) in glacial acetic acid. The endpoint may be determined potentiometrically using a glass calomel electrode system or, alternatively, by adding 5 drops of Nile Blue hydrochloride indicator (1% in glacial acetic acid) to the sample and titrating to a yellow-green endpoint. Each ml of 0.1N perchloric acid is equivalent to 31.57 mg of clonazepam.

8. Acknowledgements

The author wishes to acknowledge the assistance of Dr. K. Blessel, Dr. R.I. Fryer and the photographic and graphic services departments of Hoffmann-LaRoche in the preparation of this profile.

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CYCLIZINE

Steven A. Benezra

INDEX

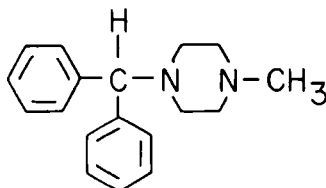
Analytical Profile - Cyclizine

1. DESCRIPTION
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor
2. PHYSICAL PROPERTIES
 - 2.1 Infrared Spectrum
 - 2.2 Nuclear Magnetic Resonance Spectrum
 - 2.3 Ultraviolet Spectrum
 - 2.4 Mass Spectrum
 - 2.5 Melting Range
 - 2.6 Differential Scanning Calorimetry
 - 2.7 Solubility
3. SYNTHESIS
4. STABILITY
5. DRUG METABOLISM AND PHARMACOKINETICS
6. METHODS OF ANALYSIS
 - 6.1 Elemental Analysis
 - 6.2 Nonaqueous Titration
 - 6.3 Thin Layer Chromatography
 - 6.4 Gas Chromatography
 - 6.5 High Pressure Liquid Chromatography
 - 6.6 Fluorimetry
 - 6.7 Colorimetry

1. DESCRIPTION

1.1 Name, Formula, Molecular Weight

Cyclizine is 1-(diphenylmethyl)-4-methylpiperazine.



$C_{18}H_{22}N_2$

Mol. Wt. 266.40

1.2 Appearance, Color, Odor

Cyclizine is a white, odorless, crystalline powder.

2. PHYSICAL PROPERTIES

2.1 Infrared spectrum

The infrared spectrum of cyclizine in KBr is shown in Figure 1. The following assignments are given to the bands in Figure 1.

3058 cm^{-1}	aromatic C-H stretch
1448 cm^{-1}	C-C skeletal vibration
1372 cm^{-1}	C-N stretch (tertiary amine)
745,698 cm^{-1}	mono-substituted benzene

Numerous other bands are in agreement with the published spectrum of N,N-dimethylpiperazine.¹

2.2 Nuclear Magnetic Resonance Spectrum

The 100 MHz NMR spectrum is shown in Figure 2. The spectrum was taken as a 3 mg/0.5 ml solution of cyclizine in CDCl_3 containing tetramethylsilane. The following assignments can be made for the observed signals.

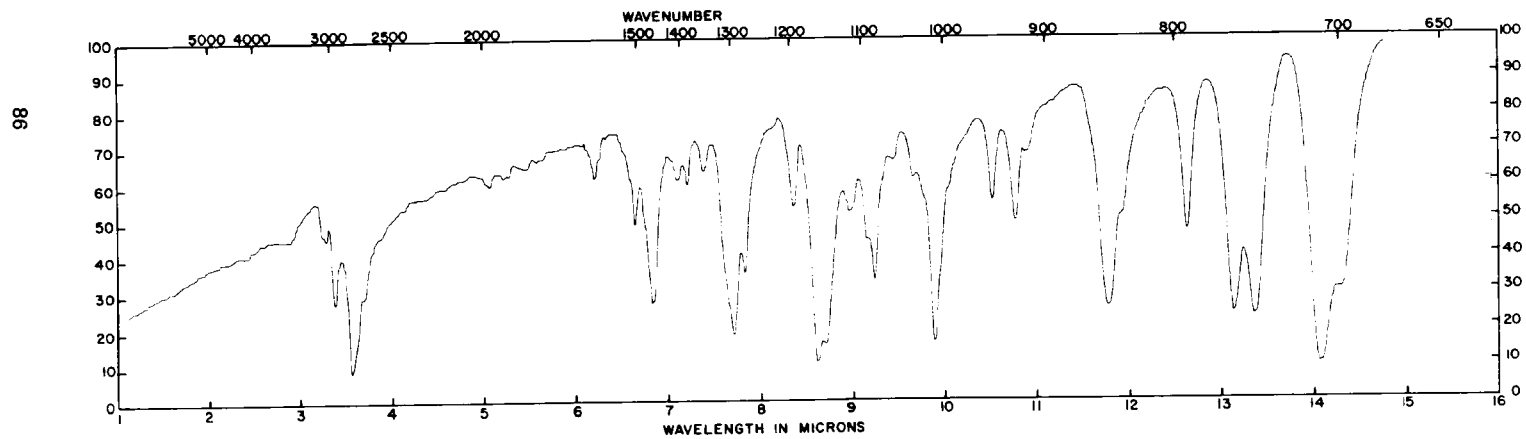


Figure 1. Infrared Spectrum of Cyclizine

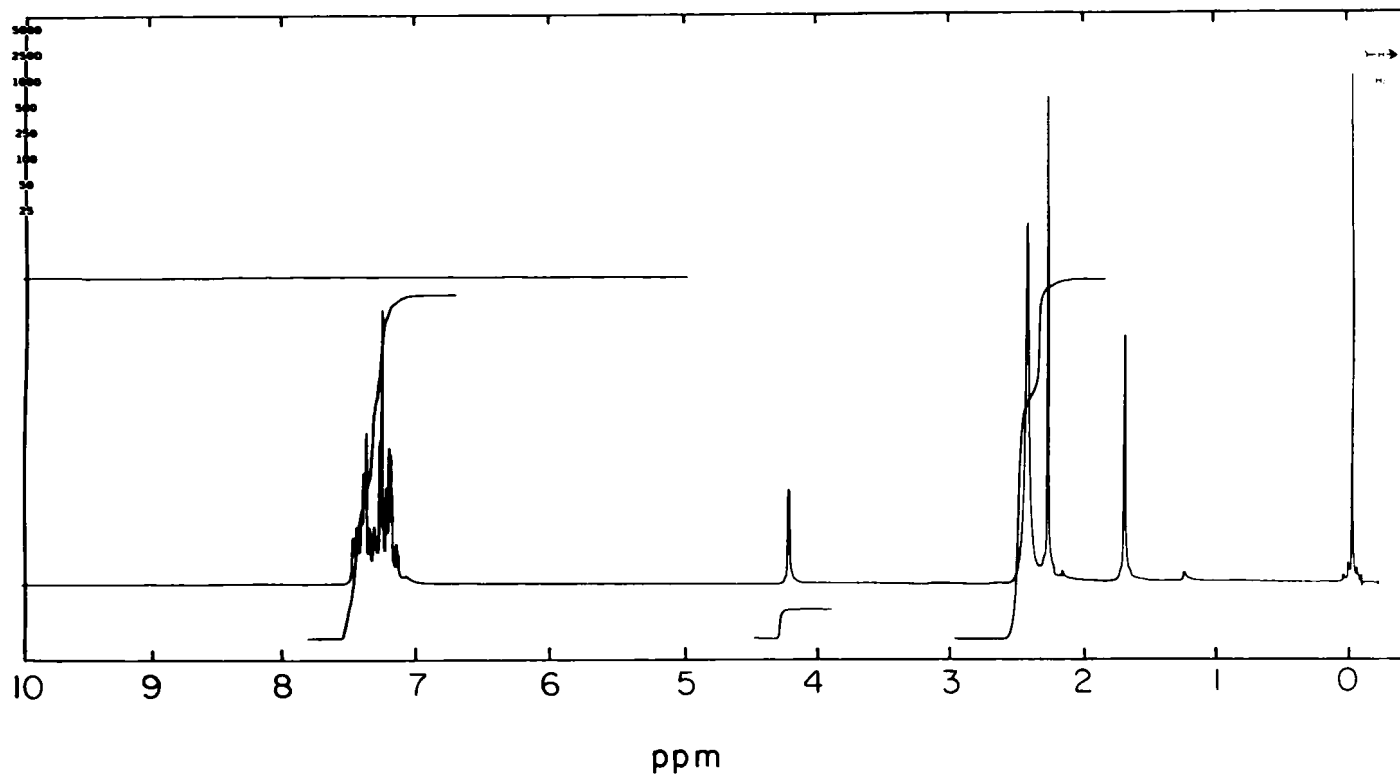
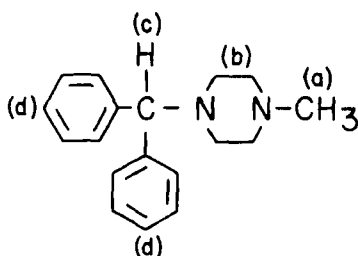


Figure 2. 100 MHz NMR Spectrum of Cyclizine

<u>Proton Position</u>	<u>No. of Protons</u>	<u>Chemical Shift (ppm)</u>	<u>Multiplicity</u>
a	3	2.27	singlet
b	8	2.43	singlet
c	1	4.21	singlet
d	10	7.19-7.44	multiplet



2.3 Ultraviolet Spectrum

The UV spectrum in 0.1 N HCl is shown in Figure 3. The maxima and minima are listed in Table 1 along with the molar extinction coefficients at the λ_{\max} . The values obtained are in good agreement with those reported by Siek.²

TABLE 1

UV Absorption Data for Cyclizine in 0.1 N HCl

<u>Wavelength of Maximum (nm)</u>	<u>Molar Absorptivity</u>	<u>Wavelength of Minimum (nm)</u>
269	540	267
263	742	260
258	694	244
253 (sh)	548	
225	1.13×10^4	

2.4 Mass Spectrum

The low resolution mass spectrum obtained at 70 ev electron energy is represented by the bar graph in

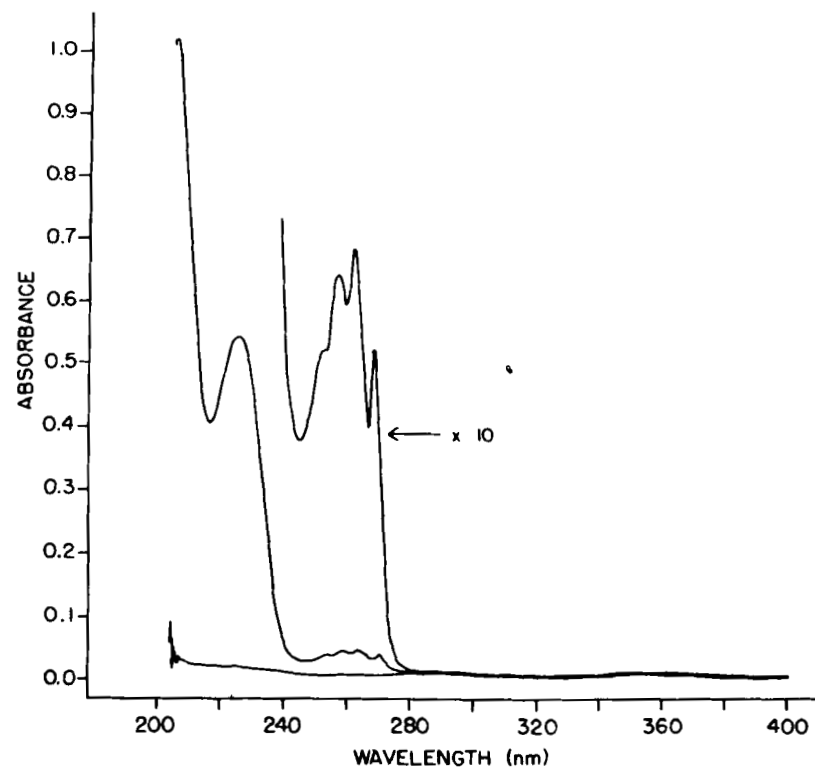


Figure 3. Ultraviolet Absorption Spectrum of Cyclizine

Figure 4. The molecular ion of m/e 266 is present but is not the base peak. The base peak in the mass spectrum occurs at m/e 99, the N-methyl piperazine fragment. The species at m/e 167 is the molecular ion minus the N-methyl piperazine radical. Ions at m/e 194, 195, 207, and 208 are from the rearrangement and fragmentation of the N-methyl piperazine moiety.

2.5 Melting Range

The melting range reported in the N.F. XIV for cyclizine is 106°C to 109°C using the class I procedure.³

2.6 Differential Scanning Calorimetry

The DSC scan for cyclizine is shown in Figure 5. An endotherm caused by melting was observed at 103°C (uncorrected) when the temperature program was 10°/minute. The ΔH_f was 7.1 kcal/mole.

2.7 Solubility

The solubility of cyclizine at 25°C is as follows:⁴

<u>Solvent</u>	<u>Solubility gm/ml</u>
Water	<0.1 mg/ml
Ethanol	0.17
Chloroform	1.1
Ether	0.17

3. SYNTHESIS

Cyclizine may be synthesized by the reaction scheme shown in Figure 6. Diphenylcarbinol is reacted to give the benzhydryl chloride which in turn is reacted with N-methyl piperazine to give cyclizine.⁵

4. STABILITY

Cyclizine is stable up to 5 years at room temperature.⁶ At 105°C cyclizine suspensions at pH 11.5 decompose⁷ to N-methylpiperazine, benzhydrol and benzophenone.

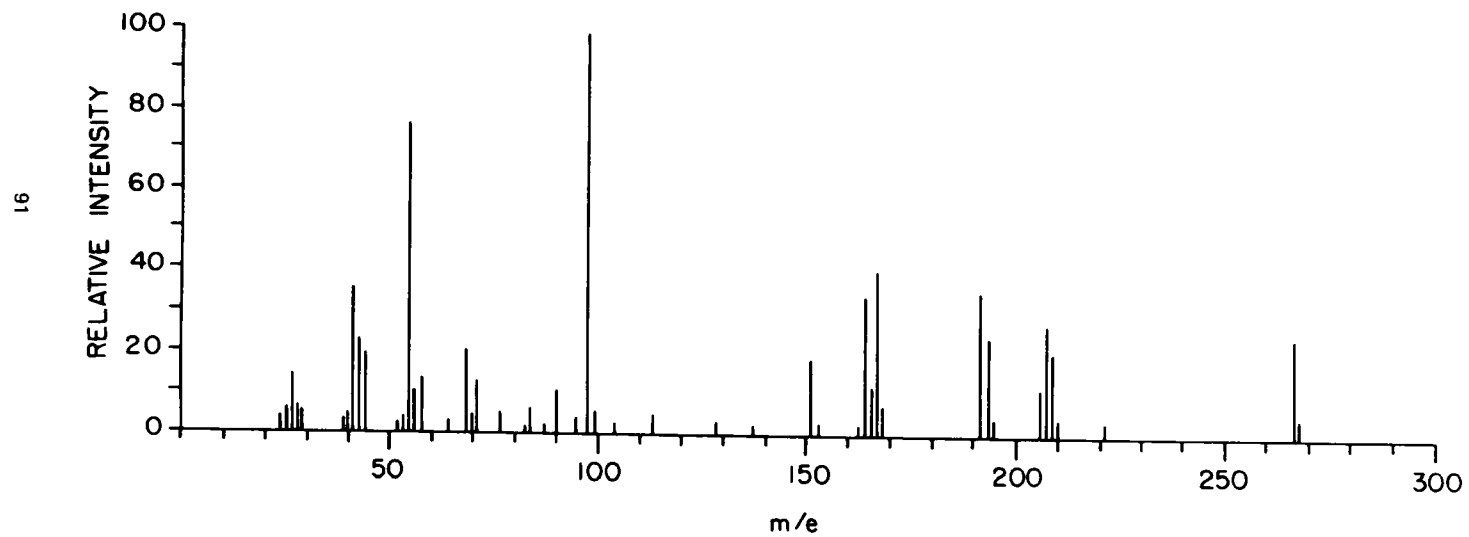


Figure 4. Mass Spectrum of Cyclizine

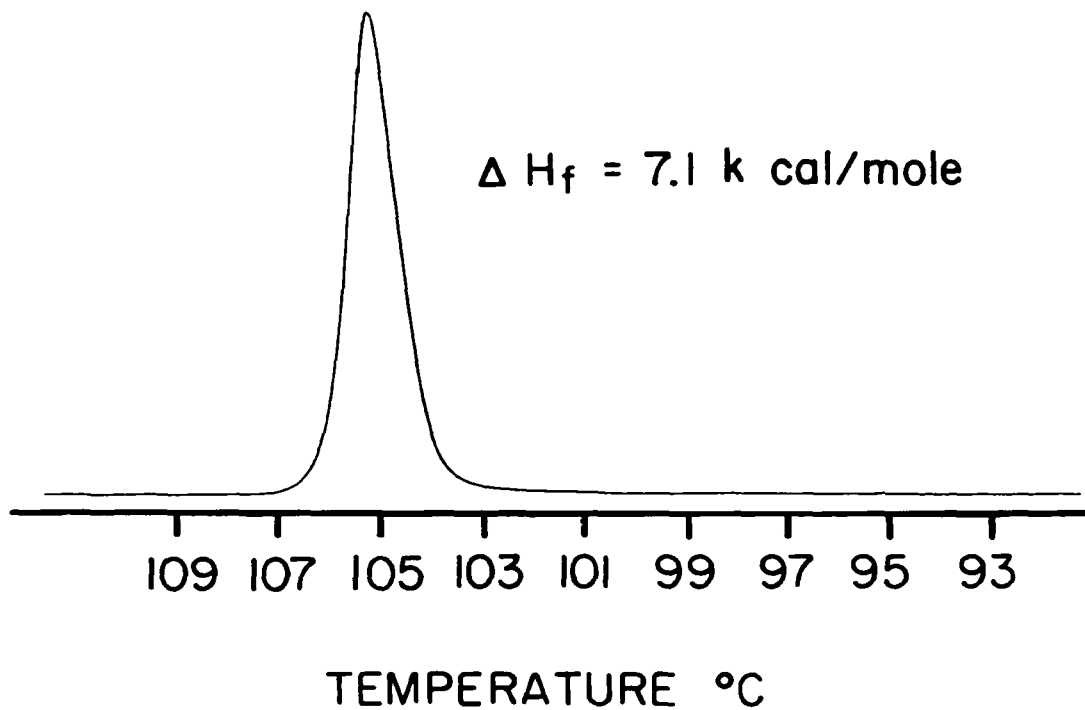


Figure 5. DSC Thermogram of Cyclizine

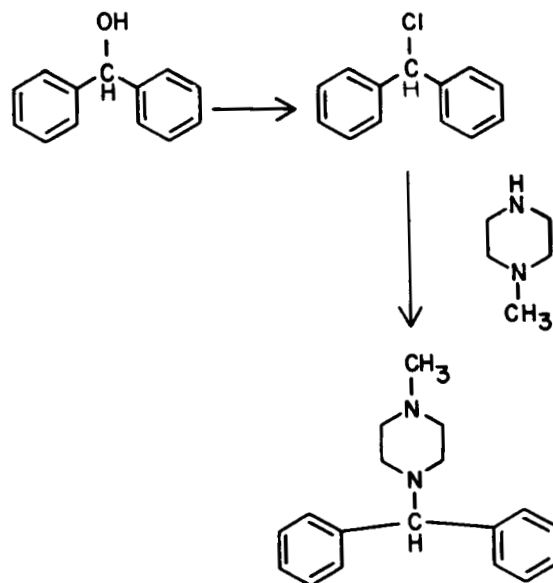


Figure 6. Synthesis of Cyclizine

5. DRUG METABOLISM AND PHARMACOKINETICS

Kuntzman and coworkers⁸ have determined that cyclizine is metabolized to its demethylated derivative, norcyclizine, which has little activity compared to cyclizine. Both the parent drug and its metabolite, norcyclizine, are distributed in plasma and tissues. The highest concentrations of drug and its metabolite were found in lung, spleen, liver, and kidney. The average half-life of norcyclizine in man was indicated to be less than 1 day when cyclizine was administered 50 mg t.i.d. for 6 days.⁹

6. METHODS OF ANALYSIS

6.1 Elemental Analysis

	<u>Theoretical (%)</u>	<u>Found (%)</u> ¹⁰
C	81.33	80.93
H	8.32	8.33
N	10.52	10.50

6.2 Nonaqueous Titration

Dissolve 0.3 g in 75 ml glacial acetic acid. Titrate with 0.1 N perchloric acid using crystal violet indicator. Each ml of 0.1₃N perchloric acid is equivalent to 0.01332 g of cyclizine.

6.3 Thin Layer Chromatography

A variety of thin layer chromatographic systems have been used for cyclizine. They are given in Table II. All visualization was done with short wave UV.

TABLE II

Thin Layer Chromatograph Systems for Cyclizine

<u>Adsorbent</u>	<u>Mobile Phase</u>	<u>R_f</u>	<u>Ref</u>
silica gel	cyclohexane:diethylamine: benzene (95:15:5)	0.55	11
silica gel	benzene:ethanol:NH ₄ OH (95:15:5)	0.61	11
silica gel	methanol:chloroform (1:2)	0.60	11
silica gel	ethylacetate:methanol:NH ₄ OH (17:2:1)	0.67	11
silica gel	chloroform:isopropyl alcohol: 5% aq. NH ₄ OH (74:25:0.6)	0.45	11
0.1 M NaOH coated SiO ₂ plates	cyclohexane:benzene: diethylamine (75:15:10)	0.55	12
0.1 M NaOH coated SiO ₂ plates	methanol	0.46	12
0.1 M NaOH coated SiO ₂ plates	acetone	0.27	12
0.1 M KHSO ₄ coated SiO ₂ plates	methanol	0.41	12
0.1 M KHSO ₄ coated SiO ₂ plates	95% ethanol	0.16	12

6.4 Gas Chromatography

Cyclizine will elute off a 2 meter 0.07% SE-30 column, a 0.08% phenyldiethanolamine succinate polymer column, a 1.07% XF1150 column, and a 1.08% Carbowax 20M column in 3.2 min, 4.9 min, 6.2 min, and 4.8 min respectively. The columns were maintained at 175°C.¹³

6.5 High Pressure Liquid Chromatography

Cyclizine as the hydrochloride salt has a retention time of approximately 6 minutes on a DuPont strong anion exchange column (37-44 μ) 1 meter x 2.1 mm i.d. A mobile phase of 0.1% sodium borate at 1 ml/min, ambient temperature is used. Detection is u.v. at 254 nm.¹⁴

6.6 Fluorimetry

Cyclizine when treated with 3% H_2O_2 solution at the 0.1 mg/ml level has fluorescence maxima at 417 and 449 nm when excited at 305 nm and 335 nm respectively.¹⁵

6.7 Colorimetry

Tissue levels of cyclizine were determined by complexation of cyclizine with methyl orange.⁸

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DIPERODON

Jordan L. Cohen

Table of Contents

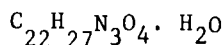
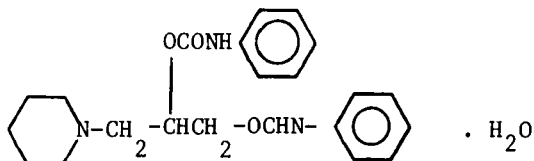
1. Description
 - 1.1 Name: Dipiperodon
 - 1.2 Formula and Molecular Weight
 - 1.3 Hydrates
 - 1.4 Salts
 - 1.5 Appearance, Color, Odor and Taste
2. Physical Properties
 - 2.1 Spectra
 - 2.11 Infrared Spectrum
 - 2.12 Nuclear Magnetic Resonance Spectrum
 - 2.13 Mass Spectrum
 - 2.14 Ultraviolet Absorption Spectrum
 - 2.2 Optical Rotation
 - 2.3 Melting Range
 - 2.4 Solubility
 - 2.5 Dissociation Constant
 - 2.6 Dipole Moment
 - 2.7 X-Ray Diffraction
3. Synthesis
4. Isolation and Purification
5. Stability and Compatibility
6. Methods of Analysis
 - 6.1 Identification Tests
 - 6.2 Quantitative Analytical Methods
 - 6.21 Elemental Analysis
 - 6.22 Ultraviolet Spectrophotometry
 - 6.23 Titrimetry
 - 6.24 Chromatography
7. Analysis in Biological Fluids and Pharmacokinetics
8. References

1. Description

1.1 Name: Diperodon

Diperodon ^{1,2,3} is designated by Chemical Abstracts as 3-piperidino-1,2-propanediol dicarbanilate monohydrate. It is also known as 1,2-propanediol, 3-(1-piperdiny1)-,bis (phenycarbamate) monohydrate.

1.2 Formula and Molecular Weight



415.49

1.3 Hydrates

Diperodon has been reported to exist in both the monohydrate and anhydrous forms with the former being the physically stable compound⁴.

1.4 Salts

The hydrochloride salt is the only reported salt of pharmaceutical interest⁵.

1.5 Appearance, Color, Odor and Taste

Diperodon occurs as a fine, white crystalline, odorless power with a characteristically bitter taste followed by a sense of numbness.

2. Physical Properties

2.1 Spectra

2.11 Infrared Spectrum

The IR spectrum of diperodon hydrochloride recorded in a KBr pellet is shown in Figure 1.⁶ Structural assignments from this spectrum are presented in Table I.

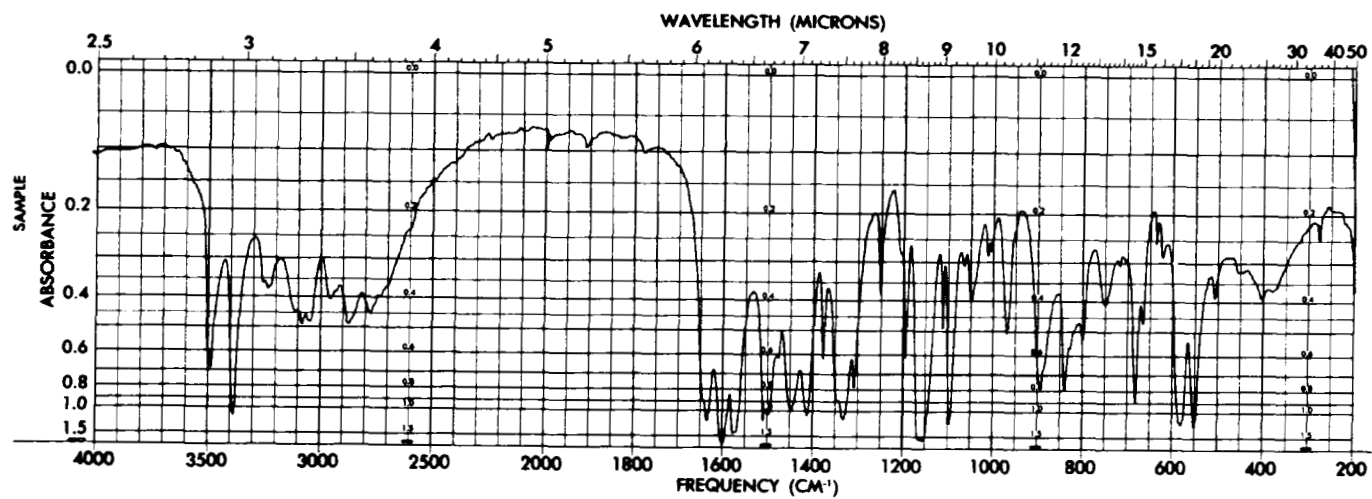


Figure 1. Infrared Spectrum of Dipiperodon Hydrochloride

Table IInfrared Spectrum of Dipiperodon HCl

<u>IR Absorption Band (cm⁻¹)</u>	<u>Assignment</u>
3400, 3200	N-H(H-bonded) stretch
2630, 2530	H-Cl, stretch
1730	C=O, stretch
1590, 1490	C=C, Aromatic, stretch
1540	N-H, bending
1200	C-O vibration
690	monosubstituted aromatic

This spectrum is consistent with the drug structure and is in good agreement with the literature infrared spectrum for dipiperodon.⁷

2.12 Nuclear Magnetic Resonance Spectrum

The 60 MHz magnetic spectrum of dipiperodon run in deuterodimethylsulfoxide is shown in Figure 2.⁶ The structural assignments are illustrated in Table II.

Table IINMR Spectral Assignments for Dipiperodon

<u>Chemical Shift (τ)</u>	<u>No.</u>	<u>Proton Assignment</u>
-CH ₂ -(aliphatic ring)	6	8.2
Impurity	-	7.5
-H-CH ₂	4	6.7
$\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{O}-\text{CH}_2- \end{array}$	2	5.7
$\begin{array}{c} \text{O} \\ \parallel \\ -\text{C}-\text{O}-\text{CH}-\text{C} \end{array}$	1	4.5
-CH-(aromatic)	5	2.7
$\begin{array}{c} \text{O} \\ \parallel \\ -\text{OC}-\text{NH}-\text{C}_6\text{H}_5 \end{array}$	2	0.1, 0.2
$\begin{array}{c} + \\ -\text{C}-\text{N}-\text{C} \\ \text{H} \end{array}$	1	-1.0

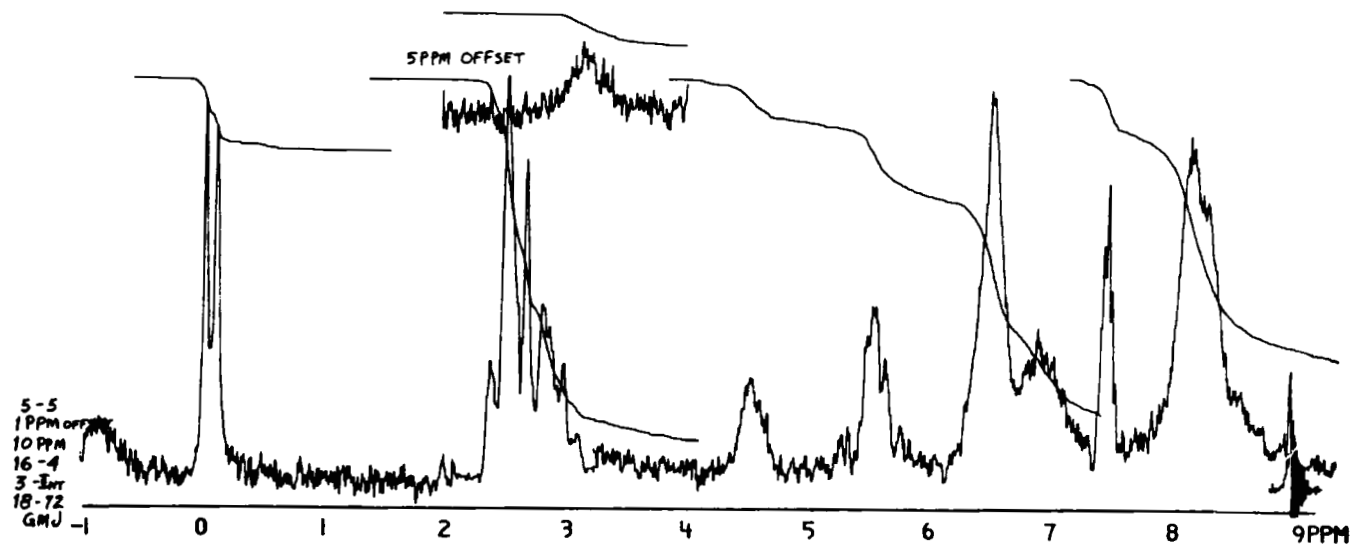



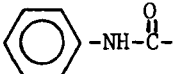



Figure 2. Nuclear Magnetic Resonance Spectrum of Diperoxon

2.13 Mass Spectrum

The low resolution mass spectrum of diperodon from a solid probe insertion is depicted in Figure 3.⁶ The extremely weak intensity of the parent ion peak at 397 m/e is typical of carbamates which undergo thermal and/or electron impact induced isocyanate elimination. Other structural assignments to this fragmentation are shown in Table III.

Table III

Mass Spectral Fragmentation of Diperodon

<u>Mass/Charge (m/e)</u>	<u>Assignment</u>
98	 N-CH ₂ -
119	 -NH-C(=O)-
124	 N-CH ₂ CHCN
141	 N-CH ₂ CH-CHOH
158	 N-CH ₂ -C=CHOH
260	loss of 119 and H ⁺

No comparative literature spectrum is available.

2.14 Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of a 2×10^{-5} M solution of diperodon in HCl is shown in Figure 4. Maximal absorption occurred at 233 nm with a molar absorptivity of 2.6×10^4 l mole⁻¹ cm⁻¹. The absorption spectrum was also recorded in heptane with a λ_{max} of 234 nm and a molar absorptivity of 3.9×10^4 l mole⁻¹ cm⁻¹. Although there is no comparative literature data the λ_{max} is in agreement with that reported for diperodon in the official assay procedure of the National Formulary!

2.2 Optical Rotation

The optical rotation of diperodon has an asymmetric center and the methyl ethyl ketone solvates of the

3419 DIPERODON HCL

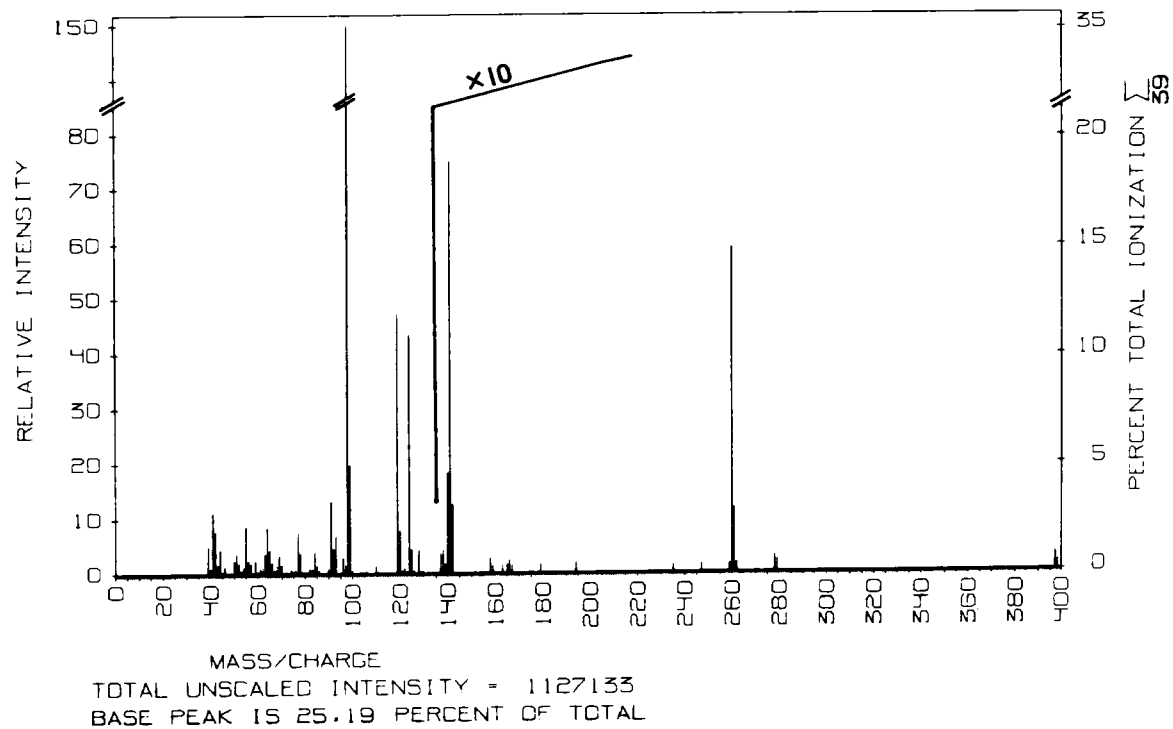


Figure 3. Low Resolution Mass Spectrum of Diperodon

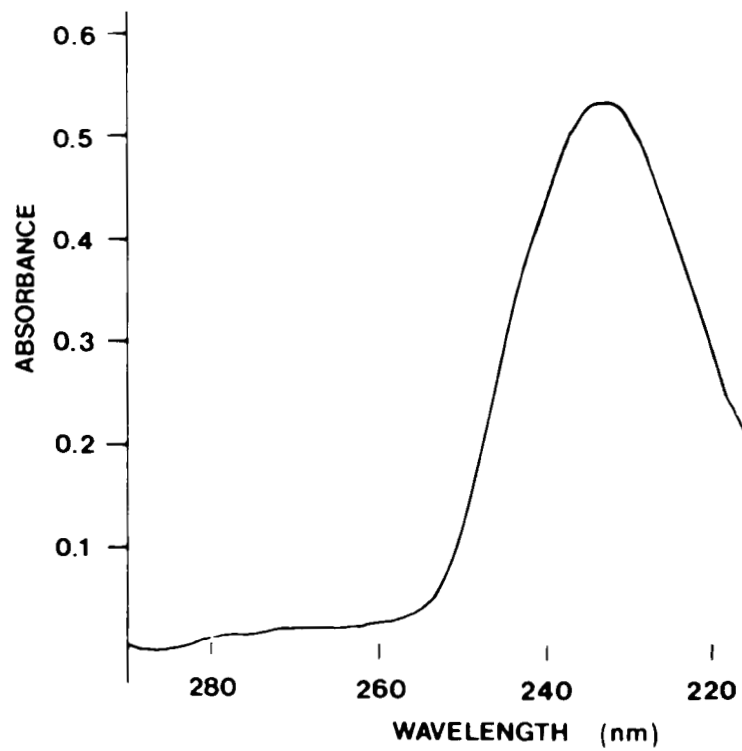


Figure 4. Ultraviolet Absorption Spectrum of Dipiperodon in 10^{-4} M HCl.

d- and l- forms were reported⁸ to be $[\alpha]_D + 14.5$ and $[\alpha]_D - 14.3^\circ$ respectively. Water was the solvent.

2.3 Melting Range

The original synthetic literature⁹ reported a melting point of 106.5°C for diperodon and a range of $197-198^\circ\text{C}$ for its hydrochloride salt. Current compendia^{3,10} list the melting range for the hydrochloride between 195 and 200°C with decomposition.

2.4 Solubility

Diperodon is practically insoluble in water but is moderately soluble in alcohol and very soluble in most non-polar solvents. The hydrochloride salt is soluble in alcohol, slightly soluble in ethylacetate, acetone and water (less than 1%) and insoluble in most organic solvents such as benzene and ether.² Its solubility in water is reportedly increased by the addition of sodium chloride.⁵ Like many other tertiary amino anesthetics, diperodon is reported to form 1:1 soluble complexes with 1,3,5-trinitrobenzene.¹² These interactions are postulated to involve the tertiary amino group and are probably charge-transfer and hydrophobic in nature. A significant spectral change is observed at 475 nm .

2.5 Dissociation Constant

Diperodon is a tertiary amine and is weakly basic. Aqueous solutions of 1% diperodon hydrochloride have a pH of 5.1.¹⁰ Although the dissociation constant is not specifically reported in the literature a pKa of 8.44 can be estimated from this information.

2.6 Dipole Moment

The dipole moment of diperodon is not available from the literature.

2.7 X-Ray Diffraction

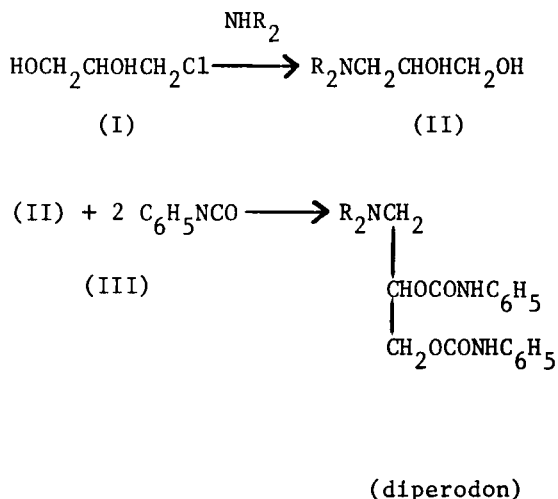
The x-ray diffraction pattern for diperodon hydrochloride has been determined and is summarized in Table IV.¹³

Table IV
X-Ray Diffraction Pattern of Diperodon HCl

2θ	I/I ₀	2θ	I/I ₀
2.26	-13	4.29	-50
2.95	-16	4.58	-23
3.13	-34	5.10	-50
3.22	-18	5.89	-60
3.49	-27	7.06	-23
3.64	-28	9.39	-22
3.94	-25	11.42	-100

3. Synthesis

Diperodon is one of several phenylurethane derivatives of dialkyl amino alcohols which have demonstrated significant local anesthetic activity.¹⁴ The original synthesis,^{9,15} which has been patented,¹⁶ involves the condensation of piperidine with glycerol chlorohydrin (I) in the presence of alkali and then condensation of the resulting 1-piperidinopropane-2,3-diol (II) with phenylisocyanate (III). The synthesis is outlined below. NHR_2 is piperidine.



4. Isolation and Purification

Diperodon is generally available as the hydrochloride salt which can be recrystallized from a mixture of acetone and ethyl acetate. The free base can then be obtained by adding an excess of alkali to an aqueous solution of the hydrochloride salt and extracting with ether. The ether must be dried over anhydrous sodium sulfate, filtered and evaporated.

The resulting diperodon is recrystallized from high boiling petroleum ether.⁹

5. Stability and Compatibility

Diperodon hydrochloride is readily neutralized by trace quantities of alkali and solutions should be stored in non-alkaline glass containers. Even traces of alkali will lead to precipitation of the insoluble free base and generally a trace of acid is added to solutions or dilutions to insure solubility.¹² The removal of acid by filter paper can also lead to precipitation of the free base and loss of potency of diperodon hydrochloride solutions. Solutions of the hydrochloride also appear to decompose over time to produce trace amounts of aniline. This is accelerated by heating during sterilization and also by the addition of alkali.¹⁸ A maximal pH of 4.8 is recommended for diperodon hydrochloride solutions and solutions with traces of cloudiness or color should not be used. Diperodon monohydrate, which is not incompatible with traces of alkali has been utilized more recently in non-solution dosage forms including lotions and ointments.⁵

6. Methods of Analysis

6.1 Identification Tests

Diperodon has been qualitatively identified by infrared spectrophotometry.⁷ Conditions for paper electrophoresis¹⁹ and paper and thin-layer chromatography²⁰ have also been established. The x-ray diffraction pattern has also been reported¹³ (see section 2.7). Several specific chemical tests have been reported to distinguish diperodon hydrochloride from other anesthetics.⁵ A white precipitate is formed upon the addition of silver nitrate which is solubilized by excess ammonia. Addition of HCl, sodium nitrate and betanaphthol produces a white precipitate which darkens to yellow and then orange upon standing. Diperodon hydrochloride reacts with chloride to give an orange-yellow precipitate.

6.2 Quantitative Analytical Methods

6.21 Elemental Analysis⁵

Chloride is determined by gravimetric analysis following the addition of silver nitrate to an ammonia solution. Nitrogen is analyzed using a modified Kjeldahl determination. Selenium oxychloride is used in place of copper sulfate as a catalyst and a four hour, rather than two hour, digestion is used.

6.22 Ultraviolet Spectrophotometry

The official assay procedure for diperodon ointment involves a chromatographic separation of the vehicle from the drug using an alumina column and a 1:1 mixture of hexane and isopropyl alcohol as the eluant. Quantitation is performed by measuring the ultraviolet absorption at 235 and 300 nm. The substantial molar absorptivity of diperodon allows a theoretical sensitivity in the low microgram /ml range to be achieved.

6.23 Titration

The compendial assay for diperodon involves titration in acetic acid using perchloric acid and crystal violet as the indicator. Each ml of 0.1 NHClO_4 is equivalent to 39.75 mg of diperodon. Diperodon hydrochloride can be titrated with HClO_4 in acetic acid following the addition of mercuric acetate to produce the free base.²¹ Methylviolet in monochlorobenzene is used as the indicator.

6.24 Chromatography

A quantitative thin-layer chromatographic method using photodensitometry has been reported.²⁰

7. Analysis in Biological Fluids and Pharmacokinetics

Diperodon has not been administered internally and no data concerning analysis in biological fluids, metabolism or pharmacokinetics is available from the literature.

Acknowledgement

The author would like to express his appreciation to Dr. William L. Davies of the Norwick Pharmacology Company for providing valuable data on diperodon.

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ERGOTAMINE TARTRATE

Bo Kreilgård

CONTENTS

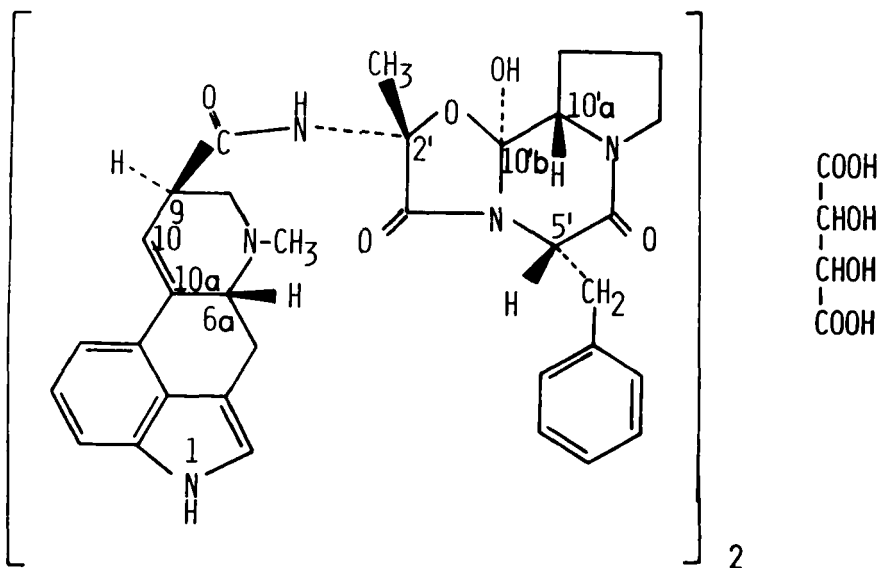
1. Description
 - 1.1 Name
 - 1.2 Formula and Molecular Weight
 - 1.3 Appearance, Color, Odor and Taste
2. Physical Properties
 - 2.1 Infrared Spectra
 - 2.2 Nuclear Magnetic Resonance Spectrum
 - 2.3 Ultraviolet Spectrum
 - 2.4 Fluorescence and Phosphorescence
 - 2.5 Mass Spectrum
 - 2.6 Optical Rotation
 - 2.7 Melting Range
 - 2.8 Solubility, Partition Coefficients and Molecular Complexes
 - 2.9 Dissociation Constants
3. Production and Synthesis
4. Degradation of Ergotamine Tartrate
 - 4.1 Chemistry of Ergotamine Degradation
 - 4.2 Stability in Pharmaceutical Dosage Forms
5. Drug Metabolism
6. Methods of Analysis
 - 6.1 Identification Tests
 - 6.2 Element Analysis
 - 6.3 Spectrophotometric Analysis
 - 6.3.1 Ultraviolet
 - 6.3.2 Colorimetric
 - 6.3.3 Fluorescence
 - 6.4 Non-Aqueous Titration
 - 6.5 Chromatographic Analysis
 - 6.5.1 Paper Chromatography
 - 6.5.2 Thin Layer Chromatography
 - 6.5.3 Column Chromatography
 - 6.5.4 High Pressure Liquid Chromatography
7. Determination in Biological Systems
8. Determination in Pharmaceutical Preparations
9. References

1. Description

1.1 Name

Ergotamine Tartrate (1-3) is the (+)-tartrate salt of (6aR,9R)-N-((2R,5S,10aS,10bS)-5-phenyl-methyl-10b-hydroxy-2-methyl-3,6-dioxo-2,3,5,6,9,10,10a,10b-octahydro-8H-oxazolo[3,2-a]pyrrolo[2,1-c]pyrazin-2-yl) 7-methyl-4,6,6a,7,8,9-hexahydro-indolo[4,3 -fg]quinoline-9-carboxamide.

1.2 Formula and Molecular Weight



$(C_{33}H_{35}O_5N_5)_2 \cdot C_4H_6O_6$ Molecular Weight: 1313,43

1.3 Appearance, Color, Odor and Taste

Ergotamine tartrate occurs as colorless crystals or white yellowish white, crystalline, odorless powder with a slightly bitter taste.

2. Physical Properties

2.1 Infrared Spectra

The infrared absorption spectrum of ergo-

ergotamine tartrate is presented in Figure 1. The spectrum was taken in a KBr pellet with a Perkin-Elmer Grating Spectrophotometer, Model 457. The IR spectrum of ergotamine base using the KBr as well as the nujol technique has been reported by Cromp and Turney (4) and Hofmann (5).

2.2 Nuclear Magnetic Resonance Spectrum

The ^1H -NMR spectrum shown in Figure 2 was obtained by dissolving ergotamine tartrate (previously dried at 60°C below 1 mm Hg for 2 hours) in deuterated dimethylsulfoxide containing tetramethylsilane as an internal reference. The spectrum was recorded on a Jeol JNM-C-60HL instrument. The spectral assignments of some of the protons are presented in Table 1. A detailed spectral analysis of setoclavine, which has a structure similar to that of lysergic acid has been reported (6). The ^{13}C -NMR spectrum of ergotamine and ergotamine have been published by Bach et al. (7).

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of ergotamine tartrate in tartaric acid solution (1% w/v) is shown in Figure 3 (8). The spectrum of ergotamine salts and ergotamine itself exhibits a characteristic flat maximum at about 317 nm and a minimum at about 270 nm. Maximum wavelengths and molar absorptivities are presented in Table 2.

2.4 Fluorescence and Phosphorescence

Ergot alkaloids of the lysergic acid and isolysergic acid type are known to exhibit fluorescence when irradiated with ultraviolet light. Loss of the 10,10a doublebond conjugated with the indole group causes loss of the fluorescence (11,14). Fluorescence spectra of ergotamine in aqueous solution (pH 2.1 and 10.8) and in ethanol are shown in Figure 4. There is a hypsochromic shift in moving from the alkaloid salt to the base and from aqueous to ethanolic solution (17,18). Heacock et al (19) described the influence of some organic solvents on the fluorescence intensity of ergotamine. The fluorescence intensity of ergotamine in

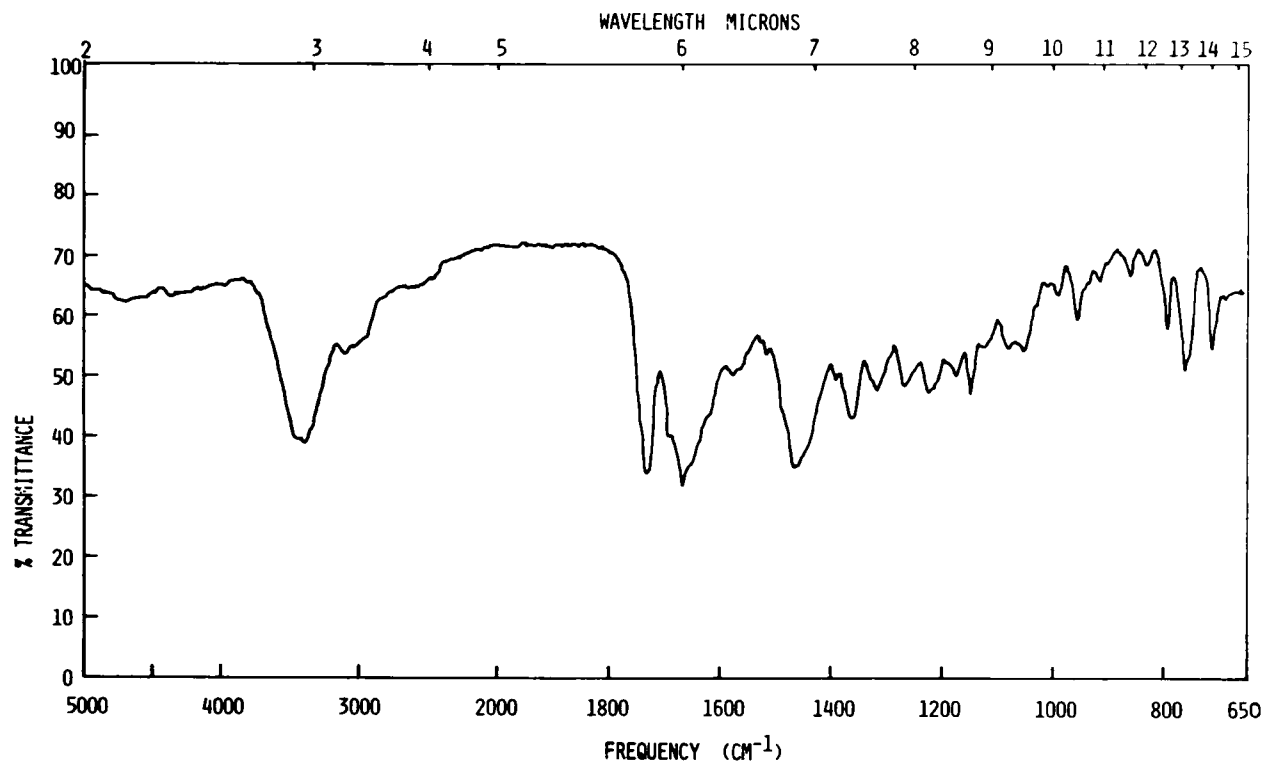


Figure 1. Infrared spectrum of ergotamine tartrate.

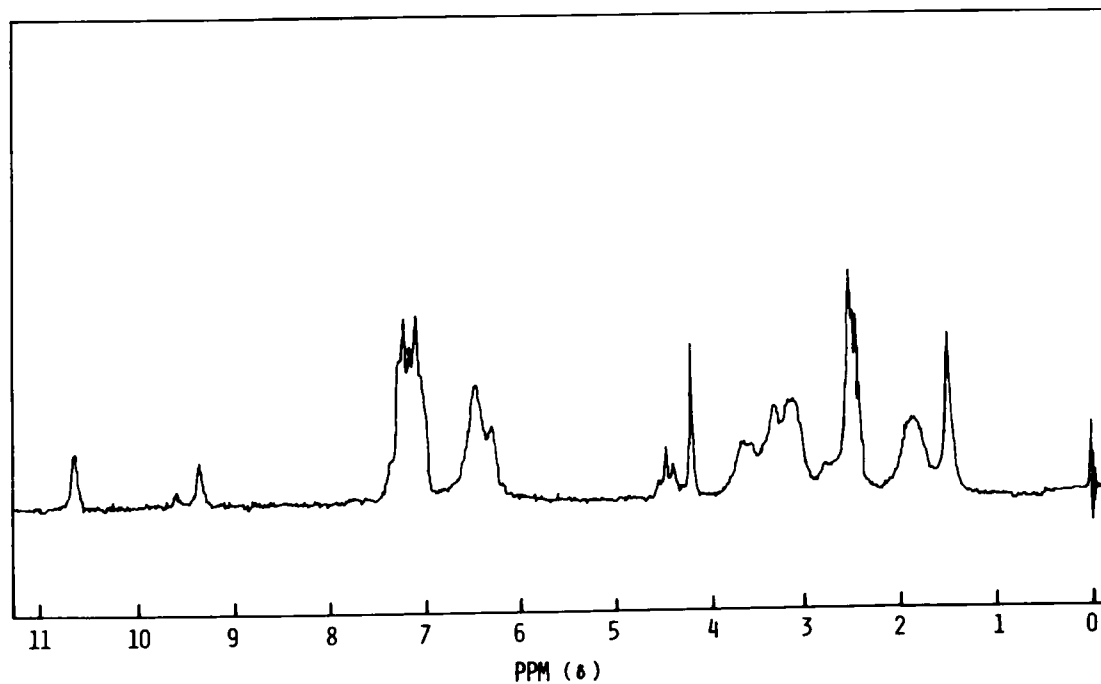


Figure 2. Nuclear magnetic resonance spectrum of ergotamine tartrate in deuterio dimethylsulfoxide.

Table 1NMR Spectral Assignments of Ergotamine Tartrate

<u>Proton</u>	<u>Number of protons</u>	<u>Chemical Shift (ppm)</u>	<u>Multiplicity</u>
NH (indole)	1 [*]	10.9	Broad singlet
NH (amide)	1 [*]	9.5	Broad singlet
Aromatic protons	10	6.9-7.4	Multiplet
$\left. \begin{array}{l} \text{C-10'b, -OH} \\ \text{>NH-CH}_3^+ \\ \text{Tartrate, -OH} \\ \text{H}_2\text{O} \end{array} \right\}$	5 [*]	6.3-6.7	
C-10'a, -H	1	6.3	Triplet
C-5', -H	1	4.5	Triplet
C-2', -CH ₃	3	1.5	Broad singlet

* Exchangeable with D₂O

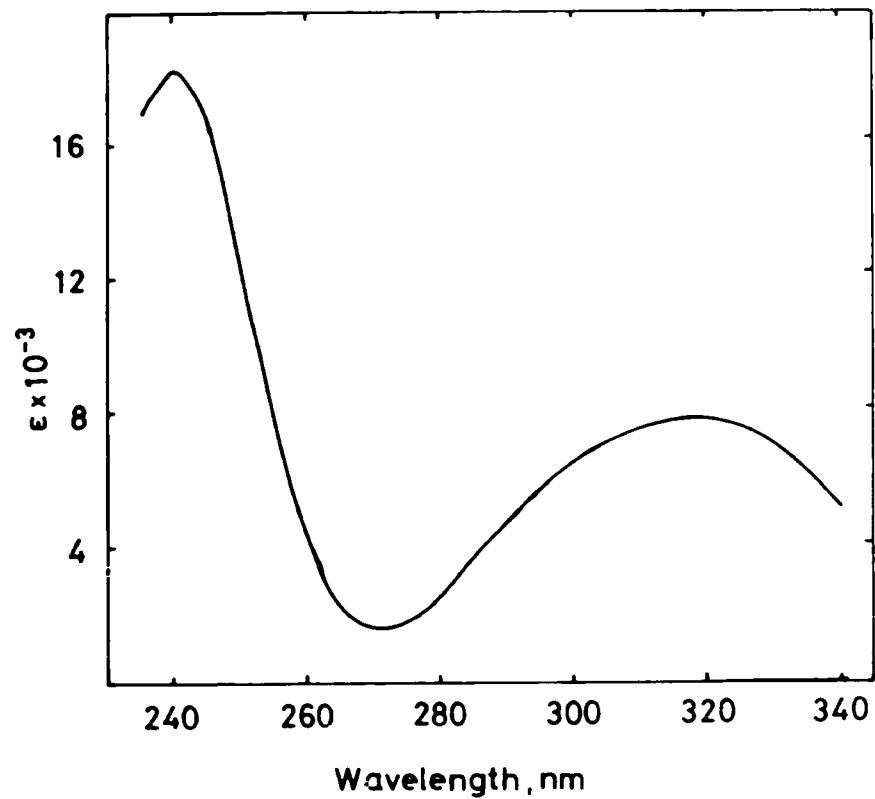


Figure 3. Ultraviolet Spectrum of Ergotamine Tartrate in 1% Tartaric Acid Solution (8).

Table 2

Ultraviolet Spectral Characteristics

<u>Compound</u>	<u>Solvent</u>	<u>λ_{max}, nm</u>	<u>$\epsilon \times 10^{-3}$</u>	<u>Reference</u>
Ergotamine tartrate	1% tartaric acid	240, 318	7.72(at 318 nm)	8
Ergotamine tartrate	1% tartaric acid	317	7.34	9
Ergotamine tartrate	0.01 N HCl	317	~7.50	2
Ergotamine tartrate	0.01 M tartaric acid	317.5	8.00	10
Ergotamine	Ethanol	318	7.24	11
Ergotamine	Methylene chloride	308-310	8.59	12
Ergotamine	Ethanol	311	8.60	13

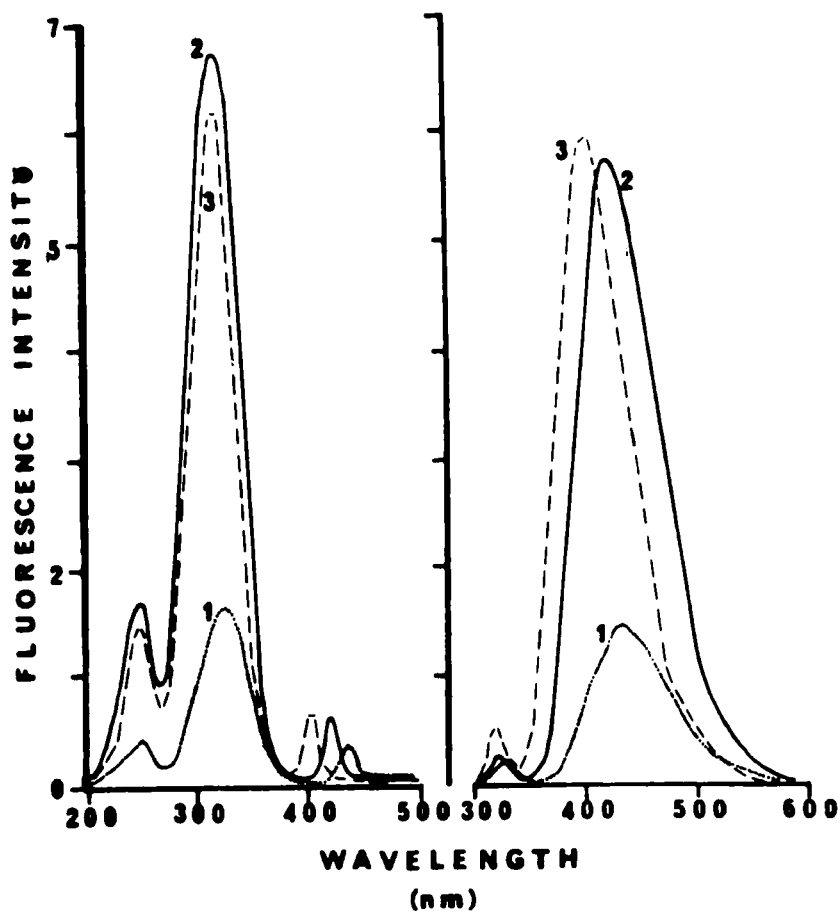


Figure 4. Excitation spectra (left) of ergotamine in: (1) water at pH 2.1 (λ_{em} 435 nm); (2) water at pH 10.8 (λ_{em} 422 nm); (3) ethanol (λ_{em} 402 nm). Emission spectra (right) of ergotamine in (1) water at pH 2.1 (λ_{ex} 325 nm); (2) water at 10.8 (λ_{ex} 318 nm); (3) ethanol (λ_{ex} 318 nm) (17).

aqueous solution is highly dependent on the pH of the solution showing almost equal intensity in the pH-range 1-9 and maximum intensity at pH ~11 (17,18). Data on fluorescence of ergotamine are summarized in Table 3.

The phosphorescence spectrum of ergotamine in ethanol at 77° K showed λ_{max} at 516, 558 and 613 nm (13).

2.5 Mass Spectrum

Several authors have reported on the mass spectrum of ergotamine (20-23). The low resolution mass spectrum of ergotamine is shown in Figure 5 (22). The molecular ion (parent peak) is absent in the spectrum obtained by 70 eV electron impact ionization (21-23), while a "reasonable-sized" parent ion is observed using high resolution mass spectroscopy at 16 eV (20). The ions b and c originate from the molecular ion by splitting of the bond between the C-9 carboxamide nitrogen and the quarternary carbon (C-2'), followed by the hydrogen atom transfer from the peptidic part to the lysergamidic part. Ion b ($m/e = 267$) is identical with the molecular ion of lysergic acid amide whose fragmentation is known (24). The substantial part of the ion current (80-90 per cent) comes from ions from the peptidic part of the molecule, while ion b and its fragments form 10-20 per cent of the total ion current (23). Other important fragments are shown in Scheme I (21, 23).

Characterization of ergotamine relative to other ergot alkaloids of the peptide type is based on the ions c, j, k and the tropylium ion since these fragments include the methyl group at C-2' and the benzyl group at C-5'.

High resolution mass spectroscopy of ergotamine has also been reported (20,21).

2.6 Optical rotation

Carbons 6a, 9, 2', 5', 10'a and 10'b of ergotamine are asymmetric, resulting in 64 possible configurational isomers. However, only isomers with changed chirality at C-9 and C-2' occur in pharmaceutical preparations. The rotation of ergotamine and some of its isomers

Table 3

Data on Fluorescence of Ergotamine

<u>Solvent</u>	<u>Temperature</u>	<u>Excitation wavelength</u> <u>(nm)</u>	<u>Maximal Emission</u> <u>wavelength (nm)</u>	<u>Reference</u>
Ethanol	25°C	320	404	13
Water	25°C	320	432	13
Ethanol	77°K	320	383	13
Water (pH 2.1)	ambient	325	435	17
Water (pH 10.5)	ambient	318	422	17
Ethanol	ambient	318	402	17
Water (pH 2-6)	ambient	335	435	18
Water (pH 8-14)	ambient	325	425	18
Acetone	ambient	350	400	19

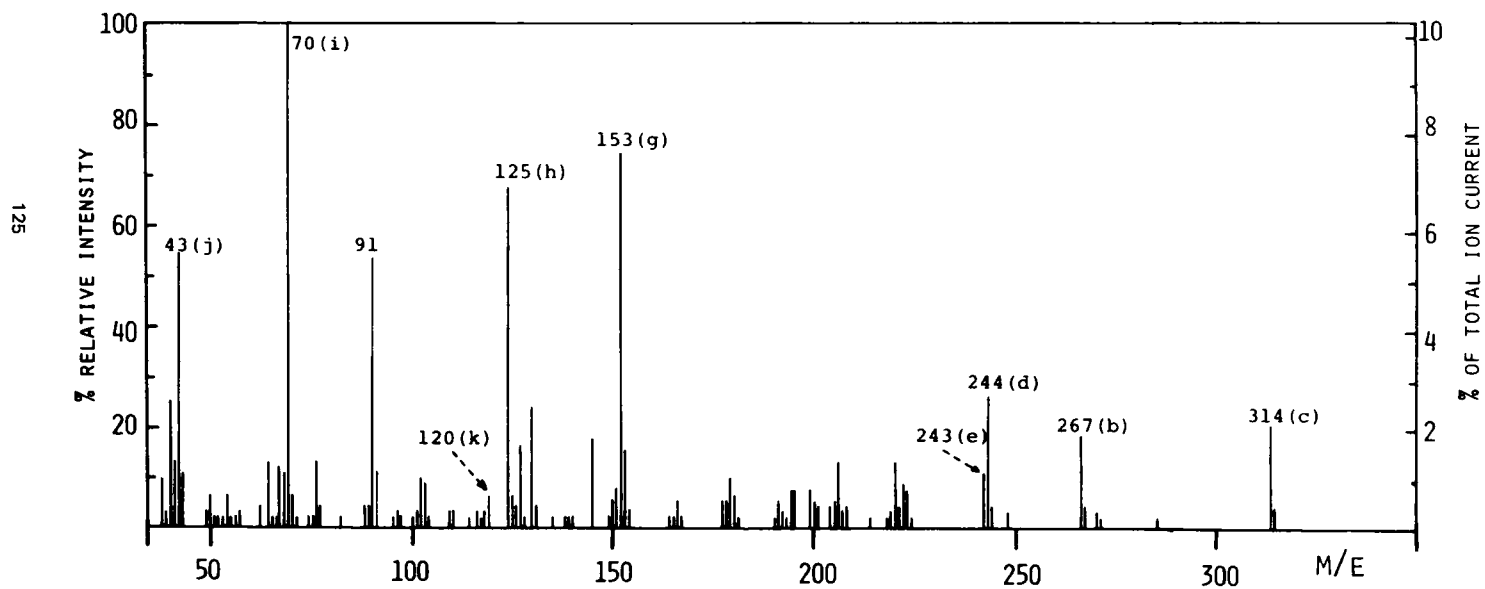
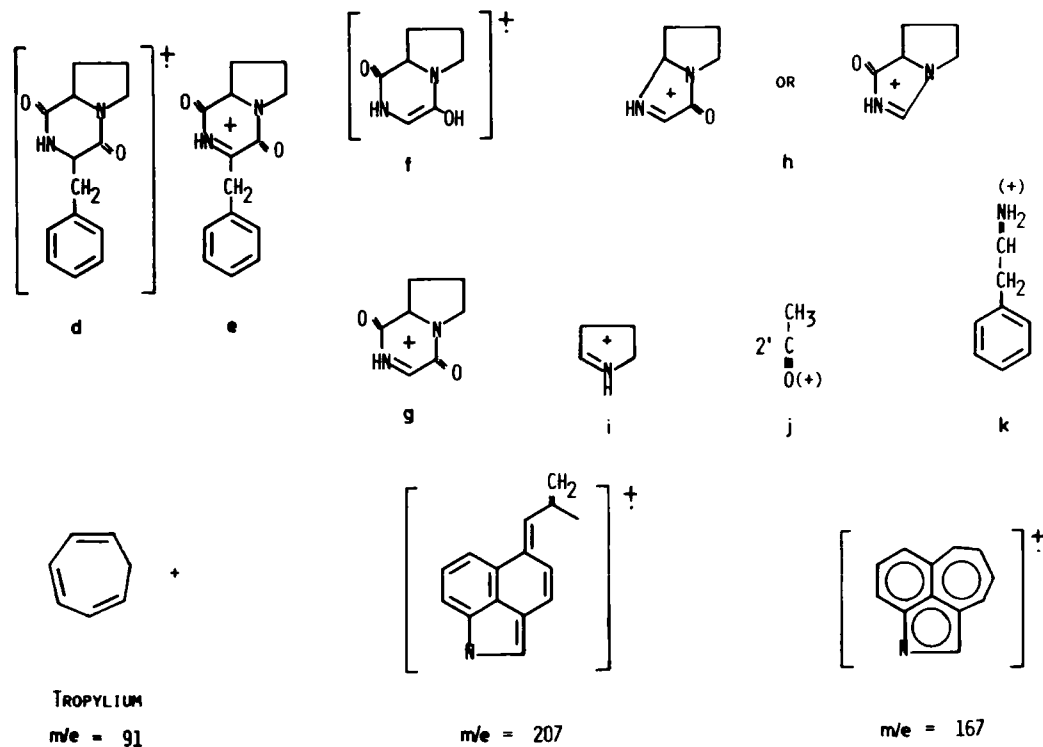


Figure 5. Mass Spectrum of Ergotamine (22).



SCHEME I Important fragments of Ergotamine on Mass Spectroscopy.

are listed in Table 4. The optical rotatory dispersion spectrum of ergotamine in methanol has been reported (25) and is reproduced in Figure 6.

2.7 Melting range

The following melting points have been reported:

180°C (decomp.)	(1)
~190°C	(2)
203°C (decomp.)	(29)

2.8 Solubility, Partition Coefficients and Molecular Complexes

The solubility of ergotamine tartrate is as follows:

Solvent	Approximate solubility mg/ml	Temperature	Reference
Water	2.5	30	(33)
"	~2	Ambient	(1,2)
0,1 N HCl	3.5	30	(33)
0,1 M phosphate buffer (pH 6.65)	0.01	30	(33)
Ethanol	2-3	Ambient	(2,34)
Chloroform	~1	Ambient	(2)

The base, ergotamine, has been reported to be soluble 1:300 in ethanol, 1:70 in methanol, 1:150 in acetone, freely soluble in chloroform and almost insoluble in water at room temperature (29).

Distribution of ergotamine between aqueous and organic solvents has been studied by several authors (17,35-37). Virtually quantitative extraction of ergotamine from aqueous alkaline solutions (pH ~8-11) into benzene, ether and chloroform has been observed (17,36). Beran and Sermonskey (38) reported on counter current distribution of ergotamine in the system of

Table 4

Optical Rotation for Ergotamine
and some of its isomers

Compound	$[\alpha]$	λ , nm	Conditions	Reference
Ergotamine	-166°	589	CHCl_3 (c=1), 25°C	26
Ergotamine	-181°	546.1	CHCl_3 (c=1), 20°C	27
Ergotamine	-150°	589	CHCl_3 (c=1), 20°C	28
Ergotamine	-160°	589	CHCl_3 (c=1), 20°C	29
Ergotamine	-192°	546.1	CHCl_3 (c=1), 20°C	29
Ergotamine	-12.7°	589	pyridine (c=1.0) 20°C	29
Ergotamine	-8.6°	546.1	pyridine (c=1.0) 20°C	29
Ergotamine	-155°	589	CHCl_3 , 20°C	30
Ergotamine	$+40^{\circ}$	589	ethanol, 20°C	30
Ergotamine	-466°	365	CHCl_3 (c=0.6), 20°C	31
Ergotamine	-375°	405	CHCl_3 (c=0.6), 20°C	31
Ergotamine	-309°	436	CHCl_3 (c=0.6), 20°C	31
Ergotamine	-174°	546	CHCl_3 (c=0.6), 20°C	31
Ergotamine	-152°	578	CHCl_3 (c=0.6), 20°C	31
Ergotamine	-145°	589	CHCl_3 (c=0.6), 20°C	31
Ergotaminine	$+450^{\circ}$	546.1	CHCl_3 , 20°C	27
Ergotaminine	$+369^{\circ}$	589	CHCl_3 (c=0.5), 20°C	29
Ergotaminine	$+462^{\circ}$	546.1	CHCl_3 (c=0.5), 20°C	29
Ergotaminine	$+397^{\circ}$	589	pyridine (c=0.5), 20°C	29
Ergotaminine	$+497^{\circ}$	546.1	pyridine (c=0.5), 20°C	29
Ergotaminine	$+381^{\circ}$	589	CHCl_3 , 20°C	30
Aci-ergotamine	-32°		pyridine (c=1.2), 20°C	32
Aci-ergotamine	$+258^{\circ}$	589	pyridine (c=1.2), 20°C	32

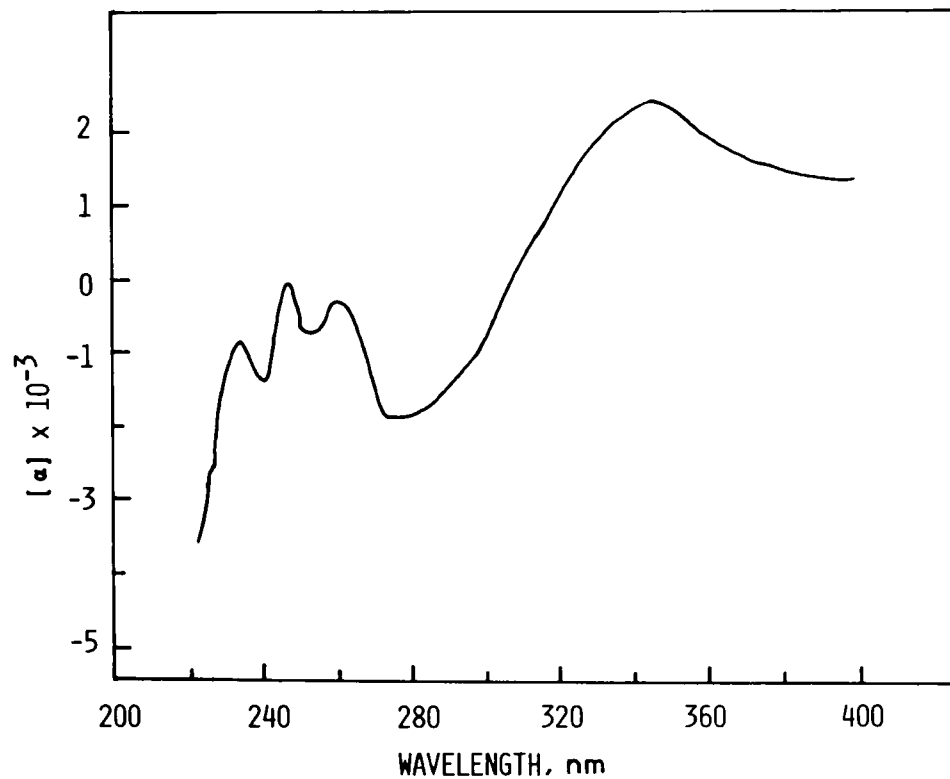


Figure 6. Optical Rotation vs. Wavelength for Ergotamine in Methanol at 25° C. (25).

aqueous tartaric acid or tartrate solutions - chloroform.

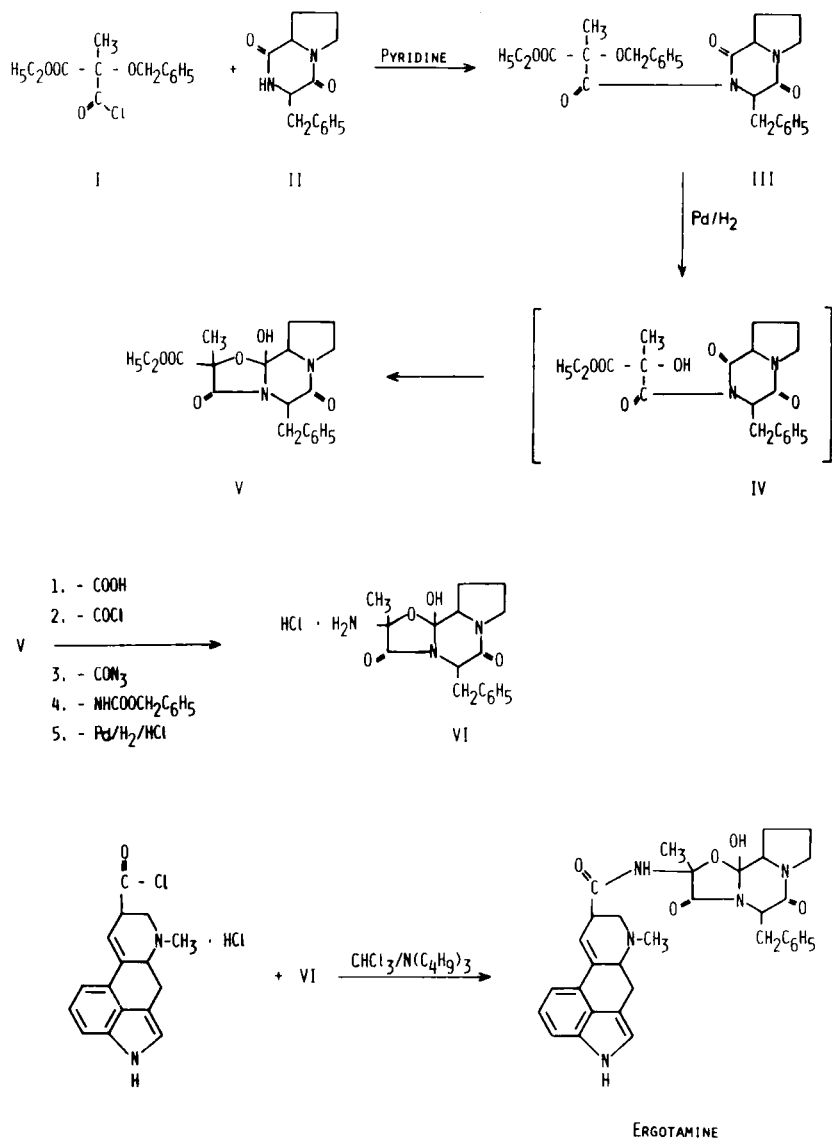
Using the phase solubility technique it has been shown that ergotamine tartrate forms molecular complexes with xanthine derivatives (33,37). The observations made do not permit calculations of stability constants.

2.9 Dissociation Constants

Due to the low solubility of ergotamine in water the acid dissociation constant, pK_a , could not be determined by conventional titration methods. An apparent pK_a value of 6.4 at 24° was obtained potentiometrically utilizing a solution of ergotamine in 2% caffeine (39) in accordance with a value of 6.3 utilizing the solubility method. A pK_a value of 5.6 in 80 per cent aqueous methylcellosolve has been reported (5).

3. Production and Synthesis

Ergotamine was originally produced by isolation of the alkaloid from the fungus *Claviceps Purpurea* (30,40). Methods of isolation of ergotamine and preparation of the tartrate salt have been described (i.e.: 5,29,42). The complete synthesis of ergotamine was not reported until 1961 (43) (Scheme II). Methylbenzyloxymalonic acid-hemi-ester (I) is reacted in pyridine with L-phenylalanyl-L-proline-lactam (II). The resulting acylated diketopiperazine (III) is very labile and is thus immediately treated with Pd/H_2 to cleave the benzyl group (IV). (IV) cyclizes spontaneously to the cyclol structure (V). Using fractional crystallization the stereoisomer with the desired chirality at C-2' is isolated. The carbethoxy group at C-2' is transformed into an amino group (VI) through a Curtius reaction. The hydrochloric salt of the peptide part is reacted with the hydrochloric salt of lysergic acid chloride (VII) in chloroform and tributylamine to form ergotamine. The first synthesis of lysergic acid was reported by Kornblum et al (44). An improved synthesis of ergotamine using (S)-(-)-methyl-benzyloxy-malonic-hemi-acid chloride has been reported (45).

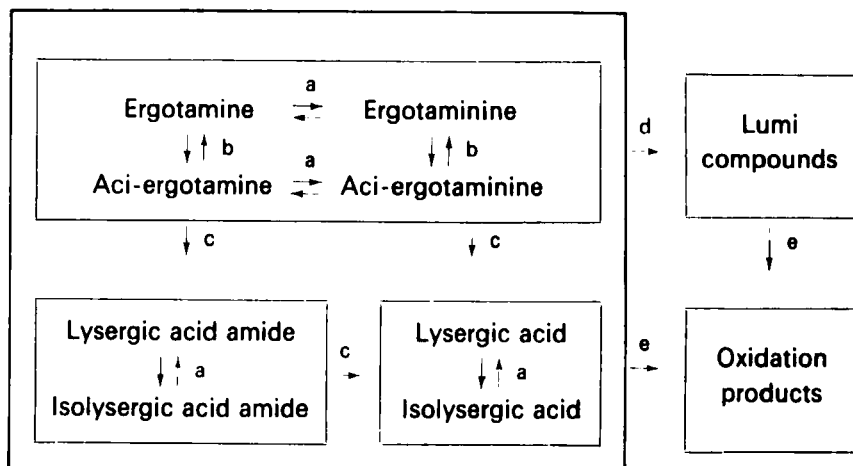


SCHEME II Synthesis of Ergotamine (43).

4. Degradation of ergotamine tartrate

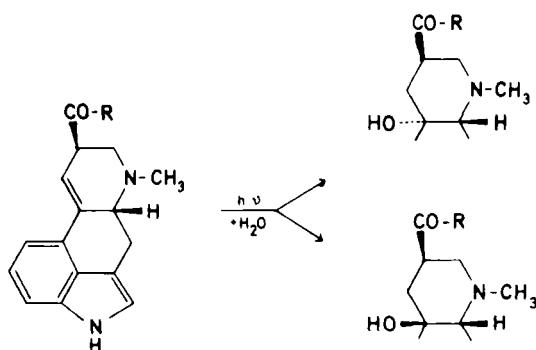
4.1 Chemistry of ergotamine degradation

The possible pathways of degradation of ergotamine tartrate are summarized in Scheme III (8).

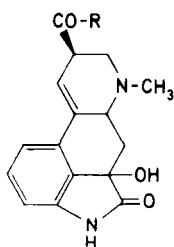


Scheme III. Degradation scheme for ergotamine. a: reversible epimerization at C-9. b: reversible epimerization at C-2' (the aci-inversion). c: hydrolysis. d: formation of lumi compounds. e: oxidation.

Epimerization at C-9 with formation of the isolysergic acid derivative, ergotaminine, is the most important route of degradation (46-48). In acidic solutions ergot alkaloids epimerize at C-2', the so-called aci-inversion (32,47,49). Hydrolysis of the four ergotamine isomers will result in formation of either lysergic acid or lysergic acid amide or the corresponding iso compounds (27,47,48,50-53). Upon exposure to light, particularly UV-light, ergot alkaloids add a molecule water to the 10, 10a-doublebond (15,55) as shown:



All compounds mentioned above are able to undergo oxidation (56). One of the expected degradation products is the 2-oxo-3-hydroxy-2,3-dihydro derivatives (R = peptide part):



Ergotamine tartrate in solid state degrades when exposed to light, humid conditions and high temperature (34).

4.2 Stability in Pharmaceutical Dosage Forms

Several studies on the stability of ergotamine tartrate in aqueous solution have been done (47,48,55,57,58,59,60,66). However, in some of the studies non-specific methods of analysis were used.

Due to the fact that epimerization at C-9 proceeds rather fast at the pH of optimal stability (47,48) injections containing the drug

are formulated to contain an equilibrium mixture of tartrates of ergotamine and ergotamine (1,2,63). In accordance with this, investigation of some liquid formulations of ergotamine showed a content of only 50-60 per cent of ergotamine (61,62). At pH = 3.6 such a mixture appears to be stable when stored protected against light in a refrigerator (48). The rate of aci-inversion increases with decreasing pH while hydrolysis into the acid or amide is at a minimum at pH ~3 (48). The influence of buffer substances on the rate of light-catalyzed formation of lumi compounds has also been investigated (55). If solutions containing ergotamine are protected against light and stored under inert gas formation of lumi compounds and oxidation are very slow processes (47,48).

Ergotamine tartrate is not stable for prolonged periods in tablets. In tablets containing ergotamine tartrate, phenobarbital and a mixture of tropane alkaloids the content of ergotamine was observed to decrease gradually during time of storage (64). In accordance with this the content of ergotamine in commercial tablets in general is less than the declared amount (61,62).

El-Shami et al (65) suggest that ergotamine tartrate in suppositories is stable for about 2 years when 4 mg tartaric acid blended with 40 mg lactose were used as stabilizing agent. The authors, however, used a method which only detected loss of active drug through oxidation.

5. Drug Metabolism

Very little information is available on the absorption, metabolism and excretion of ergotamine (66).

Ergot alkaloids of the peptide type are in general poorly and irregularly absorbed from the gastrointestinal tract and a latent period of ~30 minutes was observed (32,66). Caffeine increases the rate and extent of absorption of ergotamine tartrate and reduces the latent period (32). The alkaloid disappears very rapidly from the blood after intravenous injection (67-69). Only a minor amount of the drug is excreted in the urine, indicating detoxification by the liver (32,66,68,69). No infor-

mation on metabolism of ergotamine seems to be available in the literature.

6. Methods of analysis

6.1 Identification tests

Ergotamine tartrate can be identified by virtue of its UV, IR, NMR and fluorescence spectra, as well as its optical rotation (see section 2). Various chromatographic methods such as TLC (section 6.6.2), PC (section 6.6.1) and HPLC (section 6.6.4) provide alternate methods for purposes of identification.

A blue color is produced when 0.3 mg alkaloid is dissolved in 1.0 ml glacial acetic acid (containing 0.5 per cent Fe(III) as FeCl_3 and 0.1% glyoxylic acid) and 1.0 ml of concentrated sulphuric acid is added (5). This so-called Keller reaction which, modified slightly, is used in USP XIX (1) is based on a reaction between the alkaloid and glyoxylic acid which is almost always present as an impurity in glacial acetic acid. The van Urk reaction is based on condensation between two molecules of an ergot alkaloid and one molecule p-dimethylaminobenzaldehyde followed by a Fe(III)-catalyzed oxidation of the condensate (5,71,72). Other color tests have been reported by Clarke (73). Ergotamine tartrate has been identified by means of TLC of its ultraviolet degradation products (74) and by oscillopolarography (75).

6.2 Elemental analysis

The elemental composition of ergotamine tartrate previously dried for two hours at 60° and at a pressure below 1 mm Hg to remove water is:

Carbon	64.01%
Hydrogen	5.83%
Nitrogen	10.66%
Oxygen	19.49%

6.3 Spectrophotometric Analysis

6.3.1 Ultraviolet

The ultraviolet absorption of ergotamine tartrate can be used for quantitation (8,9,12,76,77), but the possibility of interference from related alkaloids, degradation products and excipients requires that the alkaloid be isolated from these other substances prior to measurement. The absorbance of the final sample is generally measured in the region 310-320 nm depending on the solvent (see section 2.3).

Ergotamine tartrate in a tartaric acid solution obeys Beer's law at 271 and 318 nm in the concentration range $(1-10) \times 10^{-5} \text{ M}$ (8,76). The content of native ergot alkaloids as impurities in hydrogenated alkaloids can be determined by measurement of the ultraviolet absorbance (10). By reading the absorbance at 271, 283 and 318 nm of a degraded ergotamine tartrate solution the extent of formation of lumi compounds and of oxidation products could be estimated (8).

6.3.2 Colorimetric

The most widely used colorimetric method for analysis of ergotamine is the reaction with p-dimethylaminobenzaldehyde (8,71,72,79-97). In the literature the reagent used is named as either the van Urk reagent (71), the Maurice Smith reagent (89) or the Allport reagent (80) depending on various minor modifications. Several agents have been suggested to bring about the oxidation of the condensate (see section 6.1) such as light (89,93,95), FeCl_3 (80, 93,95), hydrogen peroxide (80,96,97) and sodium nitrite (95). The absorbance of the final sample is generally measured at 550 nm. The method is not specific for ergotamine. All compounds with intact lysergic or isolysergic acid structure as well as lumi derivatives will interfere. Measuring absorbance at 546 and 586 nm following the van Urk reaction and calculations assuming a two-component system will lead to an estimate of the amount of lumi derivatives in a given ergotamine tartrate sample (8). It has been suggested to use metaldehyde reagent rather than a p-dimethylami-

nobenzaldehyde reagent due to improved sensitivity and specificity (98,99). Ergotamine tartrate has also been analysed colorimetrically by reaction with amidopyrimidine (100). Ion-pair formation between ergotamine and tropaeolin 000 (the sodium salt of 4-[(2-hydroxy-naphthyl)azo]benzol-sulphonic acid) has been used in the quantitative analysis of ergotamine (101).

6.3.3 Fluorescence

A fluorimetric analysis for ergotamine tartrate in tablets has been described by Hooper et al (17). The tablets were extracted with an acidic aqueous solution, which after being made alkaline was extracted with benzene. After evaporation of benzene the residue was dissolved in ethanol and the fluorescence intensity was read with an excitation wavelength of 318 nm and an emission wavelength of 402 nm. The minimum detectable concentration was reported to be 0.002 μg per ml and the standard curve was linear up to 5 μg per ml (17). It is reported that the standard curve for ergotamine tartrate in tartaric acid solution is non-linear in the range 10-60 μg per ml (102). In order to increase sensitivity fluorimetric detectors have been used in analysis of ergotamine by high performance liquid chromatography (19,103). For determination of ergotamine tartrate in pharmaceutical dosage forms by quantitative thin layer chromatography, elution followed by fluorimetric analysis of the eluate has been used (61). The fluorescence intensity of ergotamine is 2.5 fold greater than that of ergotamine (19). Fluorimetry has been used to determine the amount of natural ergot alkaloids in hydrogenated alkaloids e.g. dihydroergotamine (78,105).

6.4 Non-Aqueous Titration

Ergotamine tartrate dissolved either in a mixture of acetic anhydride and glacial acetic acid (1) or a mixture of dioxane and glacial acetic acid (2) can be titrated with perchloric acid in glacial acetic acid. The endpoint can be observed potentiometrically or by using

crystal violet as indicator. Each ml of 0.05 N perchloric acid is equivalent to 32.84 mg of ergotamine tartrate.

Isolation of the alkaloid base by chloroform extraction of an alkaline aqueous solution and subsequent titration with perchloric acid or p-sulphonic acid has been described (84).

6.5 Chromatography

6.5.1 Paper chromatography

Several paper chromatographic systems for ergotamine are summarized in Table 5, and methods for visualizing the spots are outlined in Table 6. Other reports on PC of ergotamine are found in references (92,106,107,159).

Quantitation of ergotamine following paper chromatography is described in references (47,108-110).

6.5.2 Thin Layer Chromatography (TLC)

A variety of TLC systems have been developed for ergotamine and most of these are summarized in Table 7. Methods used for detection and identification of ergotamine on the plate are summarized in Table 8.

Quantitation of ergotamine following TLC is done using either elution technique (8, 111-114,120) or in situ scanning (62,115-119). The most suitable solvent to use as eluting agent is a water-methanol mixture to which an inorganic or organic acid is added (112,120). Measurement of the eluate is done using either UV-spectrophotometry or colorimetry. The in situ measurement is done using UV-reflectance (116), fluorimetry (62,115,117) or transmission of plates sprayed with the van Urk type reagent (119). TLC systems have also been mentioned in references (58,78,81,85,101,111, 115,121-127).

6.5.3 Column Chromatography

Intact ergotamine and the most important degradation product ergotaminine can be determined in pharmaceuticals by using two se-

Table 5

Paper Chromatography Systems for Ergotamine

<u>No.</u>	<u>Paper</u>	<u>Impregnation</u>	<u>Solvent system</u>	<u>R_f</u>	<u>Application</u>	<u>Reference</u>
1.	Schleicher and Schüll no.2043b	Acetone-formamide (6:4)	Benzene	0.17	Separation of ergot alkaloids	12,15
2.	Whatman no.1	Dimethylphthalate	Formamide-water (4:6) (pH=5.2 with Formic acid)	0.37	Separation of ergot alkaloids	16
3.	Schleicher and Schüll no.2043b	Citric acid-phos- phate buffer (pH=5.6)	Benzene-ethanol (95%) (9:1)	0.35		41
4.	Schleicher and Schüll no.2043b	Ethanol-formamide (1:1)	Benzene	0.05		54
5.	Whatman no.1	Ethanol-formamide (1:1)	Chloroform	0.86		70
6.	Schleicher and Schüll no.2043bM	Dimethylphthalate	Formamide-0.066M Na ₂ HPO ₄ solution (4:6)	0.05	Separation from degradation pro- ducts	47
7.	Schleicher and Schüll no.2043bM	Dimethylphthalate	Formamide-citrate buffer (pH 4.4) (2:8)	0.38	Separation from degradation pro- ducts	47

<u>No.</u>	<u>Paper</u>	<u>Impregnation</u>	<u>Solvent system</u>	<u>Rf</u>	<u>Application</u>	<u>Reference</u>
8.	Schleicher and Schüll No.2043bM	Dimethylphthalate	Formamide- 0.1 N KOH (2:8)	0.00	Separation from degrada- tion products	47
9.	Schleicher and Schüll no.2043bM	Formamide-benzoic acid (25:1)	Ether	0.24	Separation from degrada- tion products	47
10.	Whatman no.1	5% sodium dihydro- gen citrate	2.4 g of citric acid in water-butanol (65:435)	0.65	Identification	78
11.	Whatman no.1	None	Methylethylketone- acetone-formic acid- water (40:2:1:6)	0.80	Identification	104
12.	Whatman no.1	None	Methylethylketone- diethylamine-water (921:2:77)	0.91		104
13.	Whatman no.1	None	10 parts of methyl isobutylketone satu- rated with 1 part of 4% formic acid	0.16		104
14.	Whatman no.1	None	10 parts of chloro- form saturated with a mixture of 1 part of methanol and 1 part of 4% formic acid	0.47		104

Table 6Visualization of Ergotamine on Paper Chromatograms

<u>No.</u>	<u>Treatment</u>	<u>Result</u>	<u>Reference</u>
1.	Ultraviolet light (λ = 254 or 366 nm)	Blue	15,78,104
2.	p-dimethylaminobenzaldehyde reagent (various modifica- tions)	Blue-violet	16,54,78, 104
3.	KMnO ₄ (1% aqueous solution)		78,104
4.	2,6-dibromoquinone-4-chloro- imide (0.5% solution in di- oxane-acetone (4:1))		104

Table 7

Thin Layer Chromatography Systems for Ergotamine

	<u>Solvent system</u>	<u>Sorbent</u>	<u>Rf</u>	<u>Application and comment</u>	<u>Reference</u>
	1. Heptane-tetrahydrofuran-toluene-chloroform (5:4:1:5)	Silica gel (Merck G), 0.2% NaOH impregnated	0.04	Separation of ergot alkaloids	128
	2. Heptane-tetrahydrofuran-toluene-ethyl acetate (10:8:3:9)	Silica gel (Merck G), 0.2% NaOH impregnated	0.14	Separation of ergot alkaloids	128
142	3. Heptane-tetrahydrofuran-toluene (2:4:5)	Silica gel (Merck G), 0.2% NaOH impregnated	0.05	Separation of ergot alkaloids	128
	4. Heptane-tetrahydrofuran-toluene (2:4:1)	Silica gel (Merck G), 0.2% NaOH impregnated	0.08	Separation of ergot alkaloids	128
	5. Heptane-tetrahydrofuran-toluene (1:4:1)	Silica gel (Merck G), 0.2% NaOH impregnated	0.16	Separation of ergot alkaloids	128
	6. Tetrahydrofuran-toluene (4:1)	Silica gel (Merck G), 0.2% NaOH impregnated	0.27	Separation of ergot alkaloids	128
	7. Benzene-cyclohexane-diethylamine (5:2:0.01)	Silica gel (Merck G), 0.2% NaOH impregnated	0.05	Separation of ergot alkaloids	128

<u>Solvent system</u>	<u>Sorbent</u>	<u>Rf</u>	<u>Application and comment</u>	<u>Reference</u>
8. Di-isopropylether-tetrahydrofuran-diethylamine (80:20:0.2)	Silica gel, formamide impregnated	0.17	Separation of ergot alkaloids	129
9. Dibuthylether-dichloromethane-diethylamine (60:40:0.2) saturated with formamide	Silica gel, formamide impregnated	0.29	Separation of ergot alkaloids	129
10. Chloroform-methanol (9:1)	Silica gel	0.39	Separation from other lysergic acid type compounds	130
11. Chloroform-methanol-concentrated ammonia (18:1:0.01)	Silica gel	0.25	Separation from other lysergic acid type compounds	130
12. Benzene-chloroform ethanol (2:4:1)	Silica gel G	0.62	Quantitative analysis	131
13. Methanol-chloroform (2:8)	Silica gel G	0.65	Identification of ergot alkaloids	132
14. Diethylamine-chloroform (1:9)	Silica gel G	0.09	Identification of ergot alkaloids	132

<u>Solvent system</u>	<u>Sorbent</u>	<u>Rf</u>	<u>Application and comment</u>	<u>Reference</u>
15. Methanol-chloroform-concentrated ammonia (20:80:0.2)	Silica gel G	0.75	Identification of ergot alkaloids	132
16. Chloroform-ethanol (96:4)	Aluminiumoxide G	0.58	Identification of ergot alkaloids	132
17. Ethylacetate-N,N-dimethylformamide-ethanol (13:1.9:0.1)	Silica gel G	0.31	Quantitative analysis	114
18. Benzene-N,N-dimethylformamide (13:2)	Silica gel G	0.31	Quantitative analysis	114
19. Chloroform-diethylether-water (7:1:2)	Aluminium oxide G	0.01	Quantitative analysis	114
20. Benzene-n-propanol-NH ₃ (1 M) (100:10:2)	Silica gel	0.29	Quantitative analysis	158
21. Chloroform-ethanol-acetone (6:4:4)	Silica gel G	0.51	Quantitative analysis	120
22. Chloroform-ethanol (9:1)	Silica gel G	0.27	Quantitative analysis	120
23. Dichloromethane-methanol (92.7:7.3)	Silica gel GF ₂₅₄ (Merck) 0.1 N Na ₂ CO ₃ impregnated	0.56	Usefulness of azeotropic mixtures in TLC	133

<u>Solvent system</u>	<u>Sorbent</u>	<u>R_f</u>	<u>Application and comment</u>	<u>Reference</u>
24. Chloroform-ethanol (92:8)	Silica gel GF ₂₅₄ (Merck) 0.1 N Na ₂ CO ₃ impregnated	0.44	Usefulness of azeotropic mixtures in TLC	133
25. Chloroform-2-butanon (17:83)	Silica gel GF ₂₅₄ (Merck) 0.1 N Na ₂ CO ₃ impregnated	0.16	Usefulness of azeotropic mixtures in TLC	133
26. Acetone-cyclohexane (67.5:32.5)	Silica gel GF ₂₅₄ (Merck) 0.1 N Na ₂ CO ₃ impregnated	0.31	Usefulness of azeotropic mixtures in TLC	133
27. Chloroform-ethanol (95%) (9:1)	Silica gel G	0.29	Separation from degradation products	61
28. Chloroform-ethanol (95%) (9:1)	Silica gel G, 1% KOH impregnated	0.16	Separation from degradation products	134
29. Benzene-chloroform-ethanol (2:4:1)	Silica gel G	0.43	Testing of purity of ergot alkaloids	135
30. Heptane-carbontetrachloride-pyridine (1:3:2)	Silica gel G	0.16	Testing of purity of ergot alkaloids	135
31. Chloroform-acetone-diethylamine (5:4:1)	Silica gel	0.24	Quantitative analysis	163

<u>Solvent system</u>	<u>Sorbent</u>	<u>Rf</u>	<u>Application and comment</u>	<u>Reference</u>
32. Chloroform-methanol (9:1)	Silica gel	0.50	Quantitative analysis	136,137
33. Chloroform-methanol (7:1)	Silica gel GF ₂₅₄	0.52	Quantitative analysis	8
34. Chloroform-ethanol (95% (9:1))	Silica gel GF ₂₅₄	0.36	Quantitative analysis	8
35. Chloroform-methanol (17:3)	Silica gel G	0.64	Quantitative analysis	112
36. Chloroform-methanol (4:1)	Silica gel G	0.75	Quantitative analysis	112
37. Morpholine-toluene (1:9)	Silica gel	0.22	Separation from other ergot alkaloid type compounds	138
38. Chloroform-methanol (9:1)	Silica gel	0.58	Separation from other ergot alkaloid type compounds	138
39. Acetone	Silica gel F ₂₅₄ (Merck, precoated)	0.32	Identification, sepa- ration from LSD	139
40. Acetone-chloroform (4:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.39	Identification, sepa- ration from LSD	139
41. Acetone-methanol (4:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.63	Identification, sepa- ration from LSD	139

<u>Solvent system</u>	<u>Sorbent</u>	<u>Rf</u>	<u>Application and comment</u>	<u>Reference</u>
42. Chloroform	Silica gel F ₂₅₄ (Merck, precoated)	0.00	Identification, separation from LSD	139
43. Chloroform-acetone (6:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.02	Identification, separation from LSD	139
44. Chloroform-methanol (4:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.62	Identification, separation from LSD	139
45. Chloroform-methanol (9:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.35	Identification, separation from LSD	139
46. Methanol-ammonia ($\rho=0.88$) (100:1.5)	Silica gel F ₂₅₄ (Merck, precoated)	0.69	Identification, separation from LSD	78,139
47. Methanol-acetate buffer (pH 4.5) (9:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.73	Identification, separation from LSD	139
48. Chloroform-cyclohexane-isopropylamine (5:5:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.11	Identification, separation from LSD	139
49. Chloroform-cyclohexane-diethylamine (5:5:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.02	Identification, separation from LSD	139
50. 1,1,1-trichlorethane-methanol (9:1)	Silica gel F ₂₅₄ (Merck, precoated)	0.20	Identification, separation from LSD	139

<u>Solvent system</u>	<u>Sorbent</u>	<u>Rf</u>	<u>Application and comment</u>	<u>Reference</u>
51. Acetone	Aluminium oxide F ₂₅₄ (Merck, precoated)	0.48	Identification, separation from LSD	139
52. 1,1,1-trichloroethane-methanol (9:1)	Aluminium oxide F ₂₅₄ (Merck, precoated)	0.52	Identification, separation from LSD	139
53. 1,1,1-trichloroethane-methanol (96:4)	Aluminium oxide F ₂₅₄ (Merck, precoated)	0.20	Identification, separation from LSD	139
54. 1,1,1-trichloroethane-methanol (98:2)	Aluminium oxide F ₂₅₄ (Merck, precoated)	0.04	Identification, separation from LSD	139
55. 1,1,1-trichloroethanol-methanol (99:1)	Aluminium oxide F ₂₅₄ (Merck, precoated)	0.00	Identification, separation from LSD	139
56. n-Butanol-citric acid-water (870 ml:4.8 g:130 ml)	Cellulose (Merck) sprayed with 5% sodium dihydrogen citrate and dried	0.77	Identification, separation from LSD	139
57. Benzene-acetone-diethyl-ether-ammonium hydroxide (25%) (4:6:1:0.3)	Silica gel G	0.65	Identification	140

<u>Solvent system</u>	<u>Sorbent</u>	<u>Rf</u>	<u>Application and comment</u>	<u>Reference</u>
58. Benzene-chloroform (4:5) saturated with formamide and mixed with 10% methanol	Silica gel G	0.41	Identification	140
59. Benzene-n-heptane-chloroform-diethylamine (40:20:30:10)	Silica gel G	0.05	Identification	140
60. Chloroform-ethanol (9:1)	Aluminium oxide	0.82	Identification	141
61. Di-isopropyl-ether-tetrahydrofuran-toluene-diethylamine (70:15:15:0.1)	Silica gel, formamide impregnated	0.11	Separation of ergot alkaloids	142
62. Dioxane-cyclohexane-diethylamine (10:20:0.5)	Polyamide	0.05	Separation of alkaloids	143
63. Chloroform-cyclohexane-diethylamine (10:20:0.5)	Polyamide	0.15	Separation of alkaloids	143
64. 2-Butanon-cyclohexane-diethylamine (20:30:0.5)	Polyamide	0.11	Separation of alkaloids	143
65. Ethanol-chloroform-acetic acid (20:200:0.5)	Polyamide	0.97	Separation of alkaloids	143

<u>Solvent system</u>	<u>Sorbent</u>	<u>Rf</u>	<u>Application and comment</u>	<u>Reference</u>
66. Water-ethanol-pyridine (10:0.5:0.3)	Polyamide	0.10	Separation of alkaloids	143
67. Cyclohexane-ethylacetate n-propanol-dimethylamine (30:2.5:0.9:0.1)	Polyamide	0.01	Separation of alkaloids	143
68. Water-ethanol-dimethyl- amine (88:12:0.1)	Polyamide	0.03	Separation of alkaloids	143
69. Chloroform-ethanol (10:1)	Silica gel	0.51	Quantitative analysis	113
70. Chloroform-methanol (9:1)	Silica gel G, 0.1 N NaOH impregnated	0.51	Identification	144
71. Benzene-heptane-chloro- form (6:5:3) followed by benzene-heptane (6:5)	Cellulose, formamide impregnated	0.06	Identification	145

Table 8Visualization of Ergotamine on Thin Layer Plates

<u>No.</u>	<u>Treatment</u>	<u>Result</u>	<u>Reference</u>
1.	p-dimethylaminobenzaldehyde Reagent (various modifications)	Blue-violet	58,78,113, 120,132, 135,137,138 139,144
2.	Ultraviolet light (λ = 254 or 366 nm)	Blue	8,78,120, 124,128, 132,138, 139
3.	<u>In situ</u> scanning		62, 115, 116,117, 119
4.	Iodine vapor		143
5.	Dragendorff Reagent		58
6.	Ammoniated copper sulphate	Violet-brown spots on light green background	58,140,141
7.	Xanthidrol-hydrogen peroxide	Blue-violet	140
8.	Ninhydrin-cadmium acetate	Red-violet spots on pink background	140
9.	Iodo platinate Reagent	Grayish violet	58,143,146
10.	Potassium permanganate		78
11.	Ferric chloride-glyoxylic acid	Blue	124

parate Celite 545 columns (147,148). The first column is impregnated with sodium bicarbonate and the alkaloid bases are eluted with chloroform. The second column is impregnated with a 20% citric acid solution and ergotamine is eluted with a portion of chloroform. Ergotamine is extracted with chloroform from the extruded support which is suspended in an aqueous bicarbonate solution. The alkaloid content in each fraction is determined using the van Urk reaction. This method has been adapted by the USP XIX in the assay of ergotamine tartrate injection (1) and in analysis of tablets containing ergotamine tartrate, tropa- ne alkaloids and phenobarbital (64). The method, however, does not take the presence of other degradation products, such as aci-derivatives, lysergic and isolysergic acid amide into account.

Ergotamine and ergotamine in drugs have been determined using Celite 545 impregnated with formamide as the stationary phase and benzene-petroleum ether (9:1) as the mobile phase (149).

Ergotamine can be quantitatively separated from other ergot alkaloids using an aluminium oxide column and methylene chloride adding increasing amounts of methanol as eluting solvent (12,15). Carless (150) used columns of cellulose impregnated with a pH 3.0 McIlvane citrate-phosphate buffer and ether, adding 0.1% pyridine as mobile phase for separation of ergot alkaloids. Only about 80% ergotamine is recovered (150).

6.5.4 High Pressure Liquid Chromatography (HPLC)

Within the last few years high pressure liquid chromatographic methods for the analysis of ergotamine have been developed. Adsorption chromatography is carried out on silica gel with several different organic solvents as mobile phases (19,103,153). A reverse phase Bondapak phenyl/Corasil or μ Bondapak C₁₈ column with acetonitrile-aqueous ammonium carbonate buffer as mobile phase, permits separation of ergotamine from its degradation products (154,155). Increased sensitivity in the

analysis of ergotamine could be achieved by using picric acid as counter ion in forming an ion-pair which on the silica gel column is distributed between a stationary aqueous phase and a mobile organic phase (152). An increased sensitivity relative to common fluorimetric detectors (19,151) could be achieved by using a high-pressure Xenon arc lamp with an integral collimating mirror as excitation source (103). Also UV-detectors at 254 nm have been used (153-155) to detect ergotamine. Recently Bethke et al (156) described a reverse phase HPLC method with solvent gradient which in less than 20 minutes enabled them to determine the content of ergotamine as well as all epimerization and hydrolysis products in pharmaceutical preparations.

7. Determination in Biological Systems

Ergotamine have been analyzed in plasma by TLC followed by in situ fluorimetry (115). Kopet & Dille (69) used the van Urk reaction to determine ergotamine in blood and tissues, while analysis of ergotamine in plasma is done using HPLC equipped with a fluorescence detector (103). Bio-availability studies on ergotamine tartrate have been done monitoring plasma and urinary radioactivity after ingestion of ^3H -labelled ergotamine tartrate (32).

8. Determination in Pharmaceutical Preparations

The following methods have been applied to analysis of ergotamine tartrate in pharmaceuticals:

	<u>References</u>
Colorimetry	81,91,92,99,157
Fluorimetry	17
Paper chromatography	47
Column chromatography	64,148,149
Thin layer chromatography	8,61,62,113,115,117,158
High pressure liquid chromatography	152,156

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This profile attempts to cover the literature on ergotamine tartrate published up to June 1975.

FENOPROFEN CALCIUM

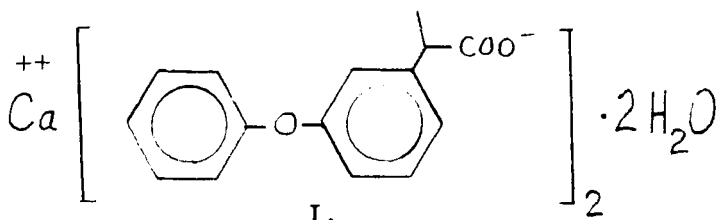
Christine K. Ward and Roger E. Schirmer

CONTENTS

1. Description
2. Physical Properties
 - 2.1 Crystal Characteristics
 - 2.1.1 Crystal Forms and Hydrates
 - 2.1.2 Melting Range and Differential Thermal Analysis
 - 2.2 Solubility
 - 2.3 pKa
 - 2.4 Electronic Spectra
 - 2.4.1 Ultraviolet Absorption Spectrum
 - 2.4.2 Optical Rotation
 - 2.5 Infrared Spectrum
 - 2.6 Nuclear Magnetic Resonance Spectrum
 - 2.7 Mass Spectrum
3. Synthesis
4. Degradation of Fenoprofen Calcium
5. Metabolism of Fenoprofen
 - 5.1 Metabolites of Fenoprofen
 - 5.2 Pharmacokinetics
6. Elemental Analysis
7. Chromatographic Methods of Analysis
 - 7.1 Thin Layer Chromatography
 - 7.2 Gas Chromatography
 - 7.3 High Pressure Liquid Chromatography
8. Titrimetric Determinations (fenoprofen and calcium)
9. Spectrophotometric Analysis
10. Analysis of Fenoprofen in Biological Samples

1. Description

Fenopropfen Calcium is calcium 2-(3-phenoxy-phenyl)propionate dihydrate (I).



Empirical Formula $(C_{15}H_{13}O_3)_2Ca \cdot 2H_2O$

Molecular Weight 558.60

It is an odorless, white, crystalline powder.

2. Physical Properties

2.1.1 Crystal Forms and Hydrates

Fenopropfen Calcium occurs as a crystalline dihydrate which is stable from 94% to less than 1% relative humidity at room temperature. Only one crystal form has been observed for the dihydrate.

2.1.2 Melting Range and Differential Thermal Analysis

When run in an open pan the thermogram of Fenopropfen Calcium (See figure 1) exhibits a large endotherm near 94°C corresponding to a loss of water accompanied by collapse of the crystal structure to a glass. When the loss of volatiles is restricted, as in a melting point tube, the endotherm appears at higher temperature (118-123°C) and is accompanied by partial liquification of the sample. This does not appear to be a true melt.

THERMAL ANALYSIS OF FENOPROFEN CALCIUM

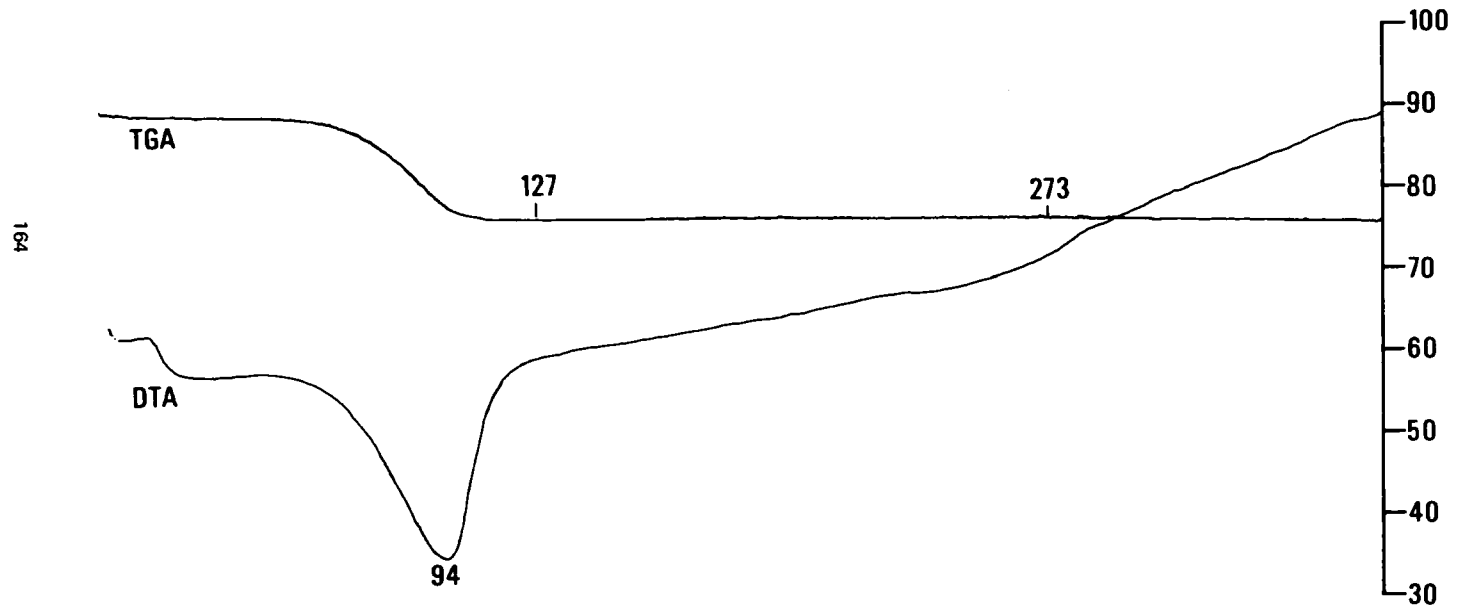


Figure 1

2.1.3 X-ray Powder Pattern of Fenopropfen Calcium

<u>d</u>	<u>I/I₁</u>	<u>d</u>	<u>I/I₁</u>
13.40	100	3.12	05
9.70	10	3.06	10
7.31	60	2.96	10
6.70	60	2.85	10
6.06	50	2.75	20
5.79	50	2.55	15
4.83	80	2.42	05
4.47	90	2.35	05
4.27	90	2.22	02
4.07	70	2.15	10
3.89	10	1.99	20
3.75	70	1.91	05
3.53	05	1.85	15
3.40	05	1.76	02
3.27	30		

2.2 Solubility

<u>Solvent</u>	<u>Solubility (mg/ml)</u>	<u>Temperature</u>
Methanol	8	37°
1-Hexanol	11	37°
Chloroform	0.01	37°
Cyclohexane	~0.01	37°
Water	2.5	25°C
Buffer pH 1.2	0.12	25°C
4.0	0.28	25°C
6.0	3.30	25°C

2.3 pKa

Water	4.5
66% Dimethylformamide/34% water	7.6

2.4 Electronic Spectra

2.4.1 Ultraviolet Absorption Spectrum

The ultraviolet spectrum of Fenopropfen Calcium in methanol is shown in Figure 2. The spectrum exhibits maxima at 266, 272, and 278 nm with $E_{1\%}^{1\text{cm}}$ values of 61.3, 70.0, and 63.2, respectively.



Figure 2. Ultraviolet Spectrum of Fenopropfen Calcium

2.4.2 Optical Rotation

Although Fenoprofen Calcium is used as the racemic mixture, optical rotations have been reported for the corresponding enantiomeric acids.¹

	$[\alpha]_D^{25} \text{ C} = 1 \text{ in CHCl}_3$
d-(+)-Fenoprofen Acid	+46.0°
l-(-)-Fenoprofen Acid	-45.7°

2.5 Infrared Spectrum

The infrared spectrum of Fenoprofen Calcium in a KBr disk is given in Figure 3. The spectrum was obtained using a Beckman IR12 Infrared Spectrophotometer. Major band assignments are as follows:

<u>Band Position, CM⁻¹</u>	<u>Assignment</u>
3660, 3600 and 3300	-OH stretching of hydrate
1560 (very strong, broad) and 1420	CO ₂ ⁻ asymmetric and symmetric stretching
1490, 1440 and 1450	aromatic ring stretching
1260 to 1210 (several bands)	C-O-C asymmetric ether stretching
930 to 695 (several bands)	primarily aromatic out of plane bending.

2.6 Nuclear Magnetic Resonance Spectrum

The 60 MHz proton nmr spectrum of Fenoprofen Calcium in deuterated dimethylsulfoxide acidified with trifluoroacetic acid is given in Figure 4. Assignments of the bands are as follows:

<u>Band Position, ppm</u>	<u>Assignment</u>
8.4-6.0 (complex multiplet)	aromatic protons
3.70 (quartet, J = 7Hz)	-CH-
1.35 (doublet, J = 7Hz)	-CH ₃

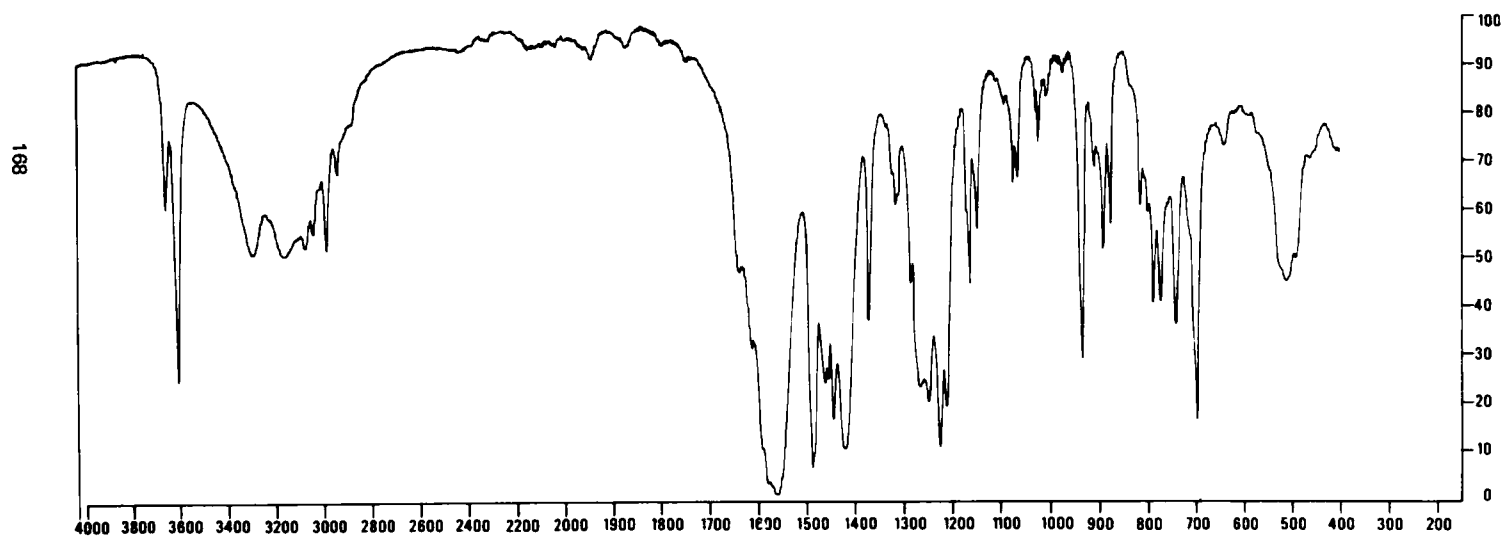


Figure 3. Infrared Spectrum of Fenopropfen Calcium

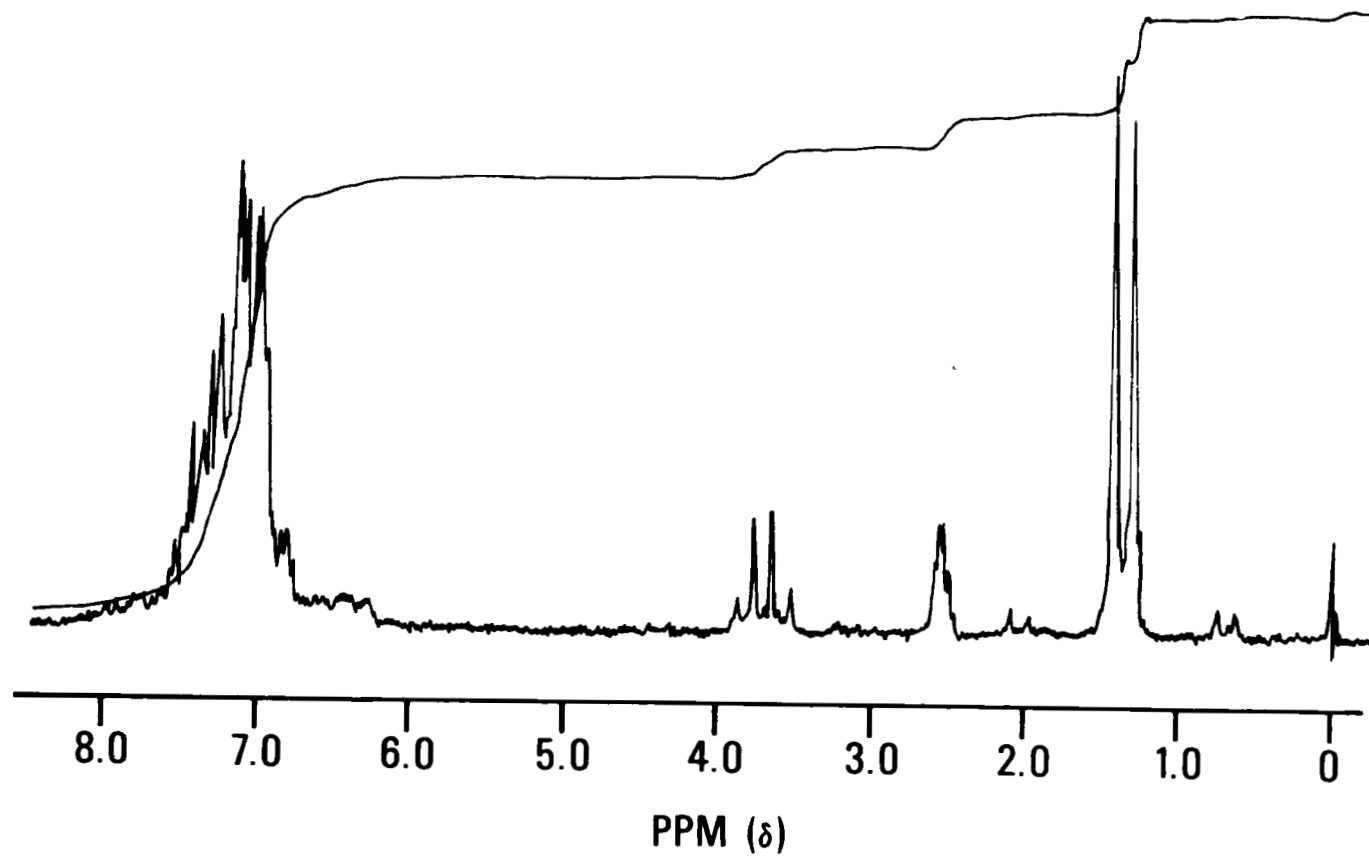


Figure 4. NMR Spectrum of Fenopropfen Calcium

2.7 Mass Spectrum

The mass spectrum of Fenoprofen is presented in Figure 5.

3. Synthesis

The synthesis of Fenoprofen Calcium is presented in Figure 6.

4. Stability

Fenoprofen is quite stable to acid, base, and heat. For example, storage at 135°C for six days results only in the loss of the waters of hydration. Samples stored for over three years at 37°C showed no degradation at all.

However, degradation of Fenoprofen can be induced by exposing aqueous solutions of the drug to intense ultraviolet light. Under these conditions photo-fries rearrangements occur leading to a mixture of the following isomeric biphenyls²:



No degradation of Fenoprofen Calcium has been observed in any formulation.

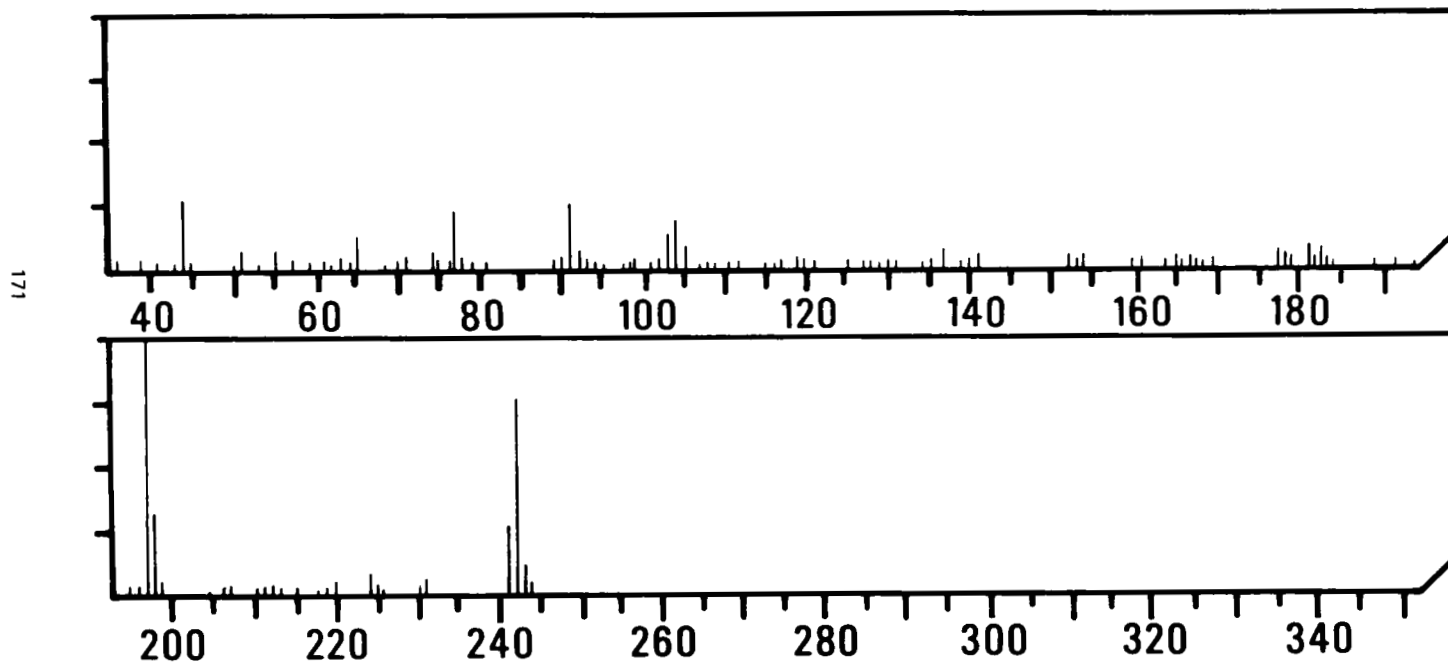


Figure 5. Mass Spectrum of Fenopropfen Calcium

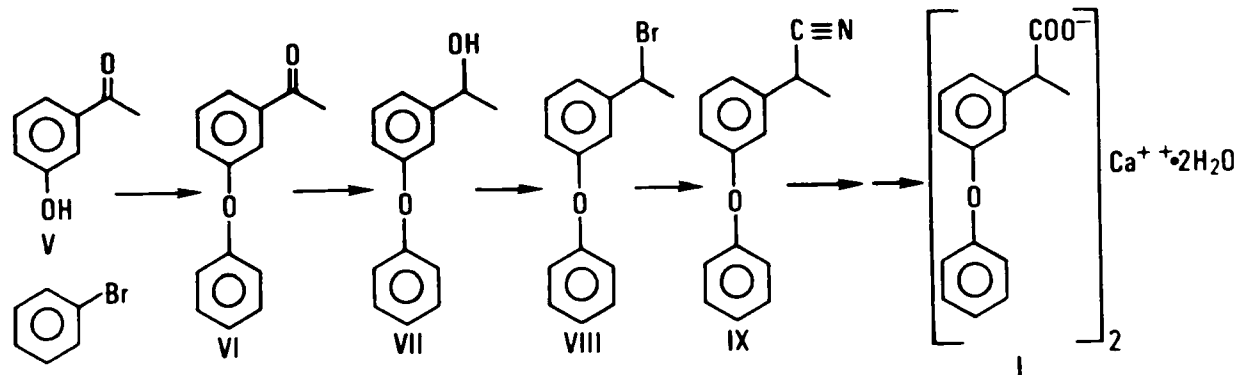
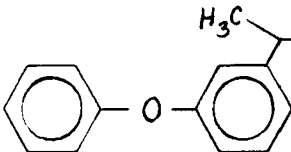
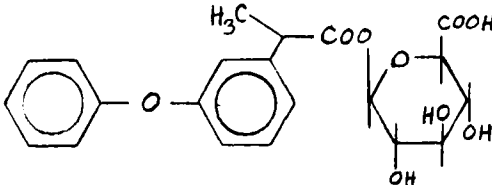
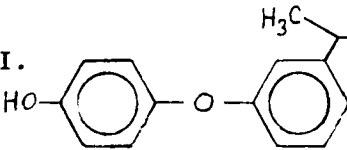
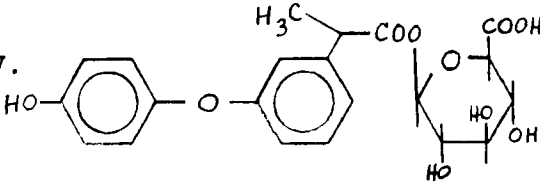


Figure 6. Synthesis of Fenopropfen Calcium

Table 1Urinary Metabolites of Fenopropfen^{3,4}

I.		3%
(unchanged Fenopropfen)		
II.		45%
III.		2%
IV.		42%
First unidentified acid labile conjugate		3%
Second unidentified acid labile conjugate		5%

5. Metabolism

5.1 Metabolites

The principle routes of metabolism of Fenoprofen involve hydroxylation of the terminal phenyl group and conjugation with glucuronic acid.^{3,4} The structures and typical percentages of the metabolites in human urine are presented in Table 1.

5.2 Pharmacokinetics

A one compartment open model provides a reasonably accurate description of Fenoprofen concentrations in plasma following oral doses.^{5,6} Representative values of the kinetic parameters for the one compartment model are given in Figure 7.⁵ Kinetic parameters have also been reported for the two compartment open model.^{5,6}

Renal clearance values for Fenoprofen range from 38.6 to 47.8 ml/min and suggest that tubular resorption of Fenoprofen occurs.⁶

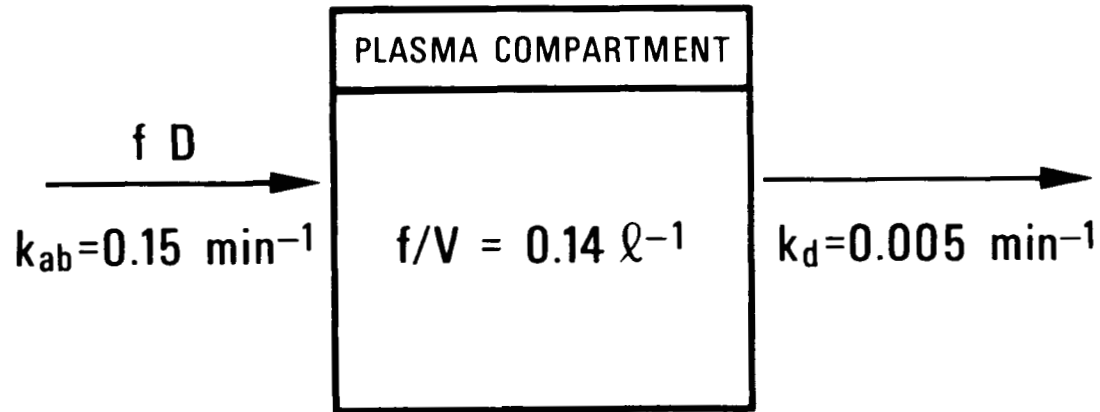
6. Elemental Analysis

<u>Element</u>	<u>Calcium Fenoprofen</u>	
	<u>Anhydrous</u>	<u>Dihydrate</u>
Ca	7.67	7.17
C	68.94	64.50
H	5.01	5.41
O	18.37	22.91

7. Chromatographic Methods of Analysis

7.1 Thin Layer Chromatography

Several thin layer systems have been reported for separation of Fenoprofen from its synthetic precursors and metabolites. These systems are summarized in Table 2. Silica gel plates were used in all cases. The Roman numerals refer to the structures given in Figure 6.



k_{ab} = absorption rate constant

k_d = elimination rate constant

fD = fraction of dose absorbed \times dose

V = volume of the plasma compartment

Figure 7. Pharmacokinetics of Fenoprofen Calcium

Table 2

 $R_f \times 100$

	<u>Solvent System</u>	<u>Compound</u>						<u>Ref.</u>
		<u>I</u>	<u>V</u>	<u>VI</u>	<u>VII</u>	<u>VIII</u>	<u>IX</u>	
1.	Hexane	0	0	0	0	12	0	7
2.	Hexane-acetic acid (98-2)	1	0	7	0	35	5	7
3.	Hexane-acetic acid (95-5)	6	0	17	3	49	15	7
4.	Hexane-acetic acid (90-10)	13	0	27	6	56	23	7
5.	Benzene	0	2	26	6	58	36	7
6.	Benzene-acetic acid (99.5-0.5)	1	2	30	9	60	40	7
7.	Benzene-acetic acid (99-1)	3	2	33	10	61	43	7
8.	Benzene-acetic acid (98-2)	6	2	33	10	65	43	7

	<u>Solvent System</u>	<u>I</u>	<u>V</u>	<u>VI</u>	<u>VII</u>	<u>VIII</u>	<u>IX</u>	<u>Ref.</u>
9.	Benzene-acetic acid (95-5)	20	6	46	20	65	52	7
10.	Benzene-acetic acid (90-10)	34	13	56	34	65	59	7
11.	Benzene-acetic acid (100-10)	37		49	30	69	56	7
12.	Benzene-acetic acid-methanol (95-5-25)	53						
13.	Chloroform	2	5	53	22	62	54	7
14.	Chloroform-acetic acid (98-2)	13	9	54	22	69	59	7
15.	Chloroform-acetic acid (95-5)	44	26	72	51	78	73	7
16.	Chloroform-acetic acid (90-10)	70	44	84	70	89	86	7

	<u>Solvent System</u>	<u>I</u>	<u>V</u>	<u>VI</u>	<u>VII</u>	<u>VIII</u>	<u>IX</u>	<u>Ref.</u>
17.	Ethyl ether	63	49	60	54	66	62	7
18.	Ethyl ether- acetic acid (98-2)	86	82	89	56	96	91	7
19.	Ethyl ether- acetic acid (95-5)	87	82	89	87	98	91	7
20.	Ethyl ether- acetic acid (90-10)	92	85	92	91	98	92	7

7.2 Gas Chromatography

Calcium Fenoprofen raw materials and formulation have been analyzed by gas chromatography. The sample is prepared by suspending the drug or crushed formulation in aqueous hydrochloric acid and extracting with chloroform, drying the chloroform over anhydrous sodium sulfate and evaporating the chloroform as necessary to concentrate the sample. The Fenoprofen acid is silylated by warming at 60°C for 15 minutes with N,O-bis-(trimethylsilyl) trifluoroacetamide and then injected onto the column. Several sets of chromatographic conditions suitable for the analysis are summarized in Table 3. Diphenamid and m-diphenylbenzene have been used as internal standards.

7.3 High Pressure Liquid Chromatography

Fenoprofen Calcium can be analyzed by high pressure liquid chromatography using the following conditions:

Column:	30 cm x 4 mm stainless steel column packed with μ -Bondapak C18.
Temperature:	Ambient (approximately 25°C)
Solvent Flow Rate:	100 ml/hr (~1100 psi)
Detector:	Ultraviolet, 280 nm
Sample Size:	Approximately 22 mcg on column
Eluting Solvent:	600 ml deionized water 400 ml acetonitrile and 20 ml glacial acetic acid
Internal Standard:	p-chlorobenzoic acid

Table 3

Conditions for Gas Chromatography
of Silylated Fenoprofen

<u>Liquid Phase</u>	<u>Solid Support</u>	<u>Length</u>	<u>ID</u>	<u>Approx. Oven Temp.</u>	<u>Approx. Retention Time</u>	<u>Ref</u>
3.8%, W98	Diatoport S	3 ft.	3 mm	175°C	4 min	8
1.0%, W98	Gas Chrom Q	2 ft.	3 mm	150°C	4 min	
0.5%, OV17	Chromosorb G- HP	4 ft.	3 mm	175°C	2 min	
1.0%, OV17	Chromosorb G- AW DMCS	3 ft.	3 mm	140°C	6.6 min.	

8. Titrimetric Determination

The carboxylate function may be determined by potentiometric titration with perchloric acid using glacial acetic acid as the solvent.

Calcium can be determined by titration with 0.05 M EDTA using Calcon indicator. About 1.5 g of Fenoprofen Calcium is dissolved in ethanol and diluted to 100 ml with ethanol. 10 ml of this solution are then titrated to the blue end-point in a solution containing 70 ml of water, 2 ml of 10% sodium hydroxide, 1 drop of 1% gelatin, 3 drops of 10% KCN and 2 drops of Calcon indicator solution.

9. Spectrophotometric Determinations

Fenoprofen acid has been determined by measuring the absorbance at 272 nm in methanol solutions acidified with acetic acid.

Fenoprofen Calcium has been determined by measuring the absorbance at 270 nm in a pH 7.5 phosphate buffer solution.

10. Analysis of Fenoprofen in Biological Samples

Fenoprofen has been determined in blood plasma samples⁸ by gas chromatography following extraction. Fenoprofen was first extracted into hexane from the acidified plasma sample, then extracted out of the hexane into 0.1N sodium hydroxide solution, and finally extracted back into hexane after adjusting the pH of the aqueous solution to about 3. The hexane was evaporated and the fenoprofen silylated using hexamethyl disilazane in carbon disulfide. The carbon disulfide solution is then injected onto a 3 ft. 3.8% W98 on Diatapore S operated at 175°C.

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ISONIAZID

Glenn A. Brewer

CONTENTS

1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor, Taste
2. Physical and Chemical Properties
 - 2.1 Spectra
 - 2.11 Infrared Spectrum
 - 2.12 Ultraviolet Spectrum
 - 2.13 Chemiluminescence
 - 2.14 Fluorescence Spectrum
 - 2.15 N.M.R. Spectrum
 - 2.16 E.S.R. Spectrum
 - 2.17 Mass Spectrometry
 - 2.2 Physical Properties of the Solid
 - 2.21 Melting Characteristics
 - 2.22 D.T.A. and D.S.C.
 - 2.23 T.G.A.
 - 2.24 Electrical Moment
 - 2.25 Electrical Conductivity
 - 2.26 Crystal Characteristics
 - 2.27 X-Ray Diffraction
 - 2.3 Solubility
 - 2.31 Water Solubility
 - 2.32 Solubility in Solvents
 - 2.4 Physical Properties of Solution
 - 2.41 pH
 - 2.42 Dissociation Constant
 - 2.43 Photolysis Constant
 - 2.44 Oxidation Potential
3. Metal Complexes
4. History, Synthesis and Manufacturing
5. Stability
6. Analytical Chemistry
 - 6.1 Identity Tests
 - 6.2 Methods of Analysis
 - 6.21 General Reviews
 - 6.22 Colorimetric Methods
 - 6.23 Spectrophotometric Methods
 - 6.24 Fluorimetric Methods
 - 6.25 Titrimetric Methods
 - 6.26 Electrochemical Methods

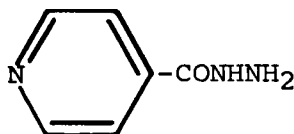
- 6.27 Gravimetric Methods
- 6.28 Microbiological and Enzymatic Methods
- 6.29 Miscellaneous Methods
- 6.3 Chromatographic Methods
 - 6.31 Paper Chromatography
 - 6.32 Thin-Layer Chromatography
 - 6.33 Ion Exchange Chromatography
 - 6.34 Other Chromatographic Methods
- 6.4 Determination of Isoniazid and its Metabolites in Body Fluids and Tissues
 - 6.41 General Reviews
 - 6.42 Colorimetric Methods
 - 6.43 Turbidimetric Method
 - 6.44 Fluorimetric Methods
 - 6.45 Electrochemical Methods
 - 6.46 Gasometric Methods
 - 6.47 Miscellaneous Chemical Assays
 - 6.48 Microbiological Assays
 - 6.49 Chromatographic Assays
- 7. Drug Metabolism
- 8. Biopharmaceutics
- 9. References

1. Description1.1 Name, Formula, Molecular Weight

Generic names - Isoniazid¹, Isonicotinic Acid Hydrazide, INH, Isonicotinoylhydrazine, Isonicotinyl hydrazide, Isonicotinylhydrazine, Tubazid, Isoniazidum.

Chemical names - 4-Pyridinecarboxylic acid hydrazide, pyridine-4-carboxyhydrazide, pyridine- γ -carboxylic acid hydrazide.

Chemical Abstracts Registry No. 54-85-3²



$C_6H_7N_3O$

Mol.Wt.137.14

1.2 Appearance, Color, Odor, Taste

Colorless or white crystalline powder which is odorless and has at first a slightly sweet and then bitter taste³.

2. Physical and Chemical Properties2.1 Spectra2.11 Infrared Spectrum

The infrared spectrum of isoniazid and other hydrazides of carboxylic acid have been recorded and band assignments were made⁴. Nagano et al⁵ in a later paper made band assignments for isoniazid, metal complexes of isoniazid and related compounds.

The infrared spectra of isoniazid as a solid in a KBr pellet and as a mull in mineral oil are shown in Figures 1 and 2. The following assignments have been made by Mrs. Toeplitz⁶.

<u>Frequency (cm⁻¹)</u>	<u>Assignment</u>
3300-3000	Bonded NH and C-H
1670	C=O
1560	Amide II
1640	NH ₂ deformation
1610 }	ring C=C and C=N
1500 }	

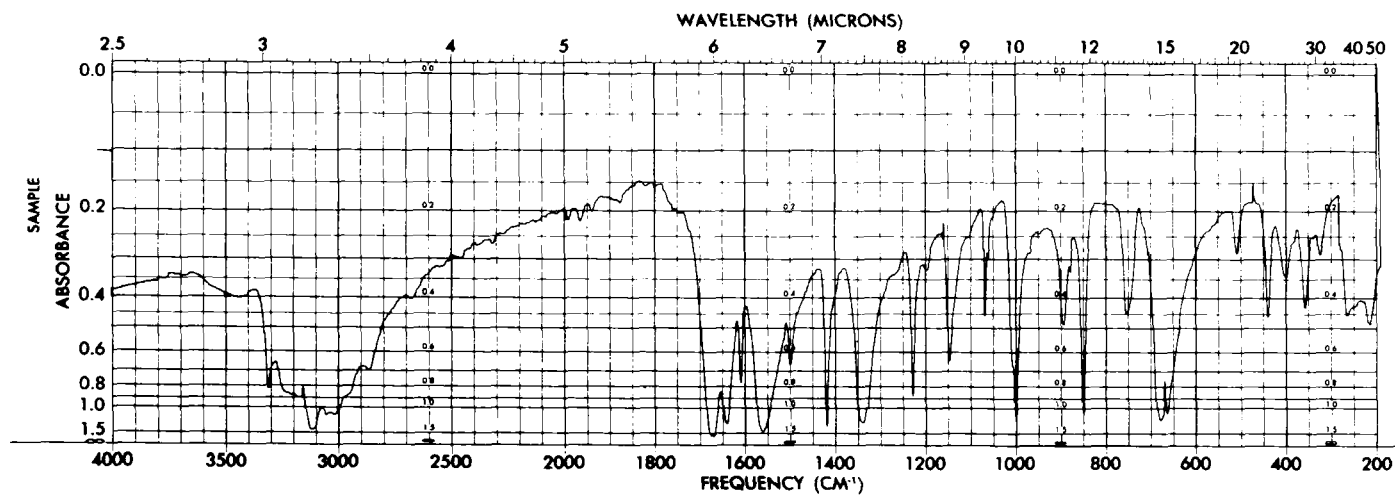


Figure 1: Infrared spectrum of isoniazid as a KBr pellet.

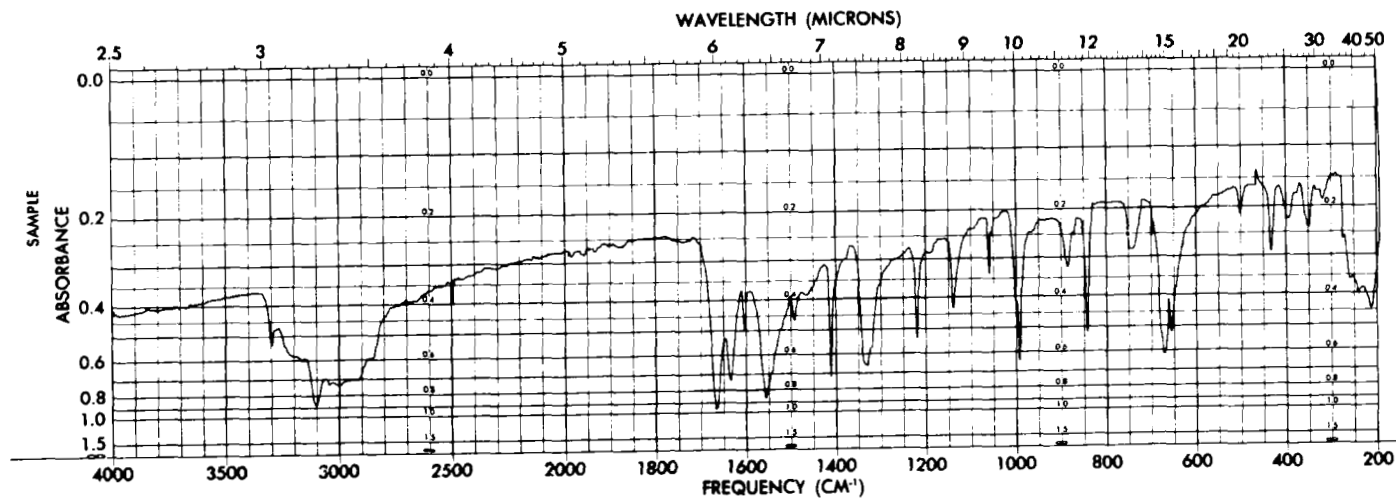


Figure 2: Infrared spectrum of isoniazid in mineral oil mull.

2.12 Ultraviolet Spectrum

Numerous authors have recorded the ultraviolet spectrum of isoniazid in a number of solvents^{7,8,9,10,11,12}. The effect of the pH of the solution on the resulting spectrum has been noted. Zommer¹³ has recorded the spectra of the hydrazones of isoniazid and acetone or p-hydroxy-benzaldehyde.

The ultraviolet spectrum of isoniazid in dilute acid (0.01N aqueous HCl) shows two approximately equal maxima at 213 nm ($E_{1\text{cm}}^{1\%}$ 437) and 265 nm ($E_{1\text{cm}}^{1\%}$ 417). The minimum occurs at 233 nm.

The spectrum in distilled water shows a broad peak at 261 nm ($E_{1\text{cm}}^{1\%}$ 306) without a defined minimum. There is a shoulder at 208 nm. In dilute alkali (0.01N aqueous alkali) the spectrum taken immediately shows a shoulder at 266 nm ($E_{1\text{cm}}^{1\%}$ 293) and peaks at 272 nm ($E_{1\text{cm}}^{1\%}$ 298) and 295 nm ($E_{1\text{cm}}^{1\%}$ 284). On standing these peaks shift so that at 2 hours there are peaks at 256 nm ($E_{1\text{cm}}^{1\%}$ 173), 262 nm ($E_{1\text{cm}}^{1\%}$ 170) and 325 nm ($E_{1\text{cm}}^{1\%}$ 76). At 24 hours the 325 nm peak disappears. The same shift takes place with higher concentrations of alkali except that it occurs more rapidly.

The ultraviolet spectrum taken in methanolic rather than aqueous solvents are similar to those in water except that the absorption maxima generally occur at slightly lower wavelengths.

2.13 Chemiluminescence

Caen¹⁵ has observed a weak chemiluminescence of isoniazid when solutions are oxidized with sodium hypochlorite. The luminescence increases with pH from 10.2 to 13. The maximum of the emission curve is at 0.552 μ corresponding to an energy of 51 Kcal. Two theories for the observed luminescence are offered, both of which depend on the presence of free OH and HO₂ radicals.

2.14 Fluorescence Spectrum

Isoniazid shows an intense fluorescence spectrum when oxidized with peroxide or after cleavage of the pyridine ring with cyanogen bromide. This fluorescence is the basis of several sensitive methods to determine isoniazid in biological materials (See Section 6.4). When a solution of isoniazid at pH 6.5 to 7.5 was treated with dilute peroxide at 100°C for 30 minutes we found the excitation maximum at 333 nm and the emission peak at 415 nm¹⁴. After isoniazid is reacted with cyanogen bromide reagent in 1.8N alkaline solution at room temperature we found an activation maximum at 312 nm and a fluorescence maximum at 392 nm¹⁴.

Isoniazid also fluoresces when reacted with certain aromatic carbonyl compounds (Section 6.24).

2.15 N.M.R. Spectrum

Several authors have studied the nuclear magnetic resonance spectrum of the hydrazides of carboxylic acid including isoniazid^{16,17,18}. Hillerbrand and co-workers¹⁹ studied the N.M.R. spectrum of the copper salt.

The N.M.R. spectra of isoniazid and D₂O exchanged isoniazid are shown in Figures 3 and 4²⁰. The 60 MHz NMR spectrum of isoniazid, in dimethyl sulfoxide-d₆ containing tetramethyl silane as internal reference shows the presence of hydrazino protons resonances at (ppm) 4.60 (broad, 2H, exchanged) and 10.15 (broad, 1H, exchanged). The aromatic protons resonances appear as multiplets at 7.73 (2H) and 8.70 (2H). (Figures 3 and 4). The complex pattern of the resonances, other than the expected doublets, suggests charge distribution in the pyridine ring. However, the hindered rotation around the N-C=O as well as C-aryl bonds can not be ruled out. The NMR spectrum in methanol -d₄ was similar to Figure 4, the hydrazino protons having been exchanged. The addition of deuterated HCl did not alter the spectrum except a downfield shift of the aromatic

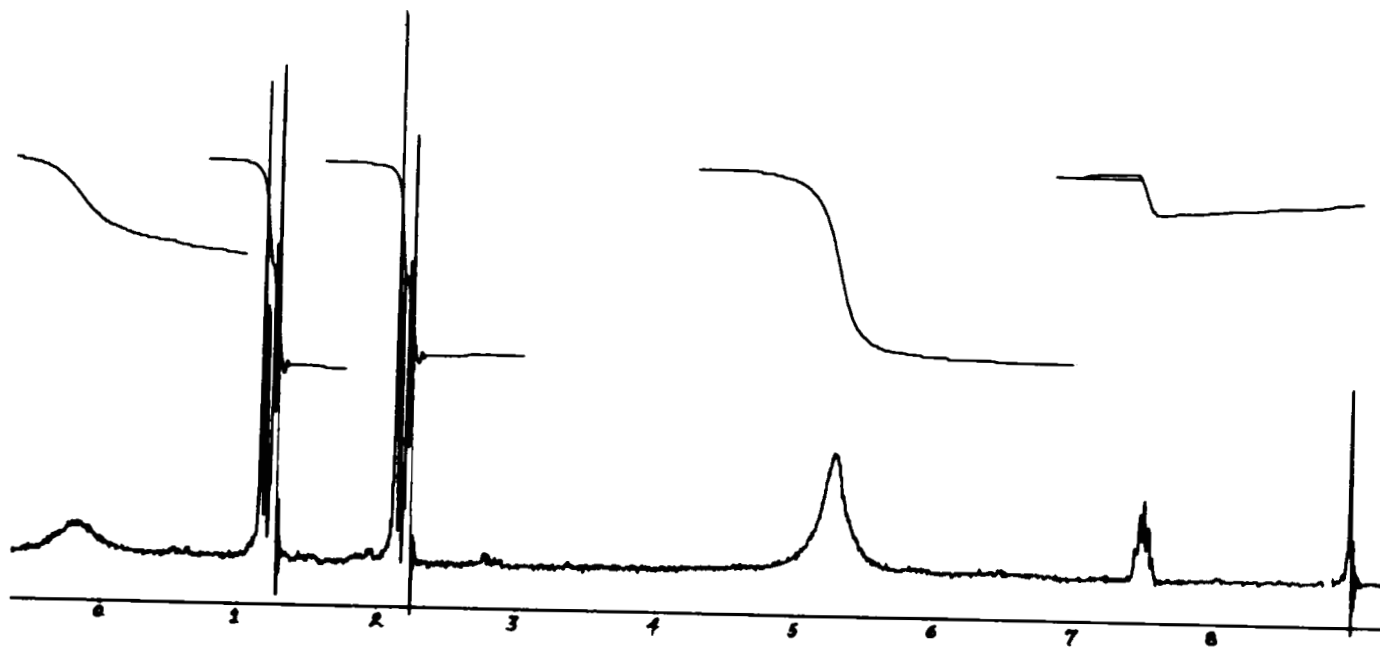


Figure 3:NMR spectrum of isoniazid in deuterodimethylsulfoxide.

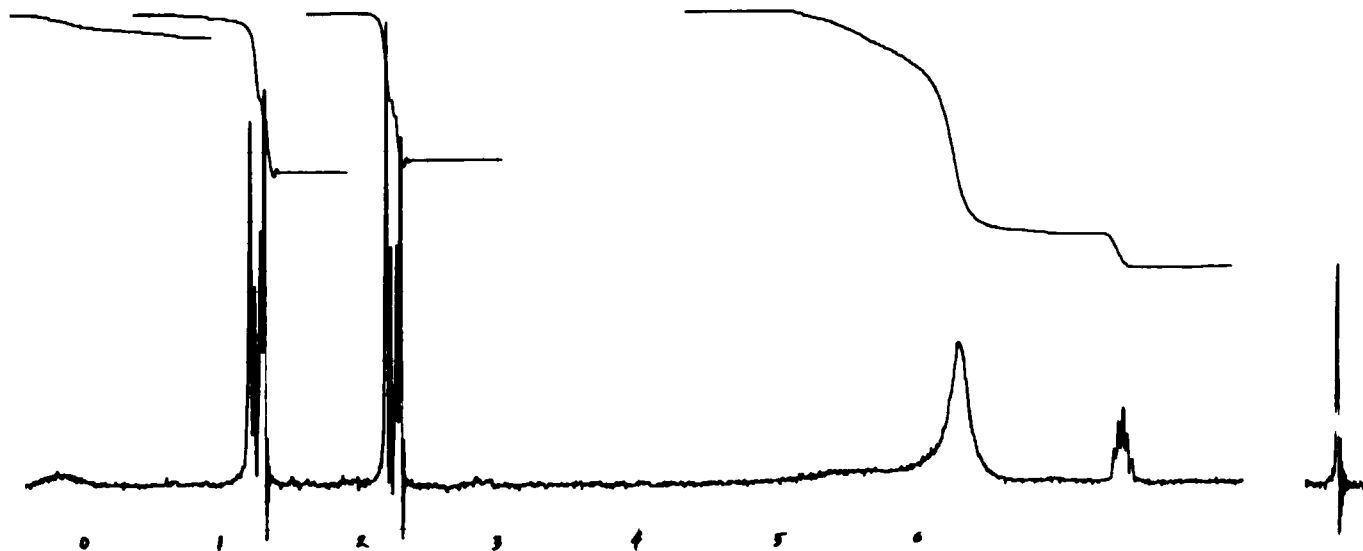


Figure 4: NMR spectrum of deuterium oxide exchanged isoniazid in deuterodimethylsulfoxide.

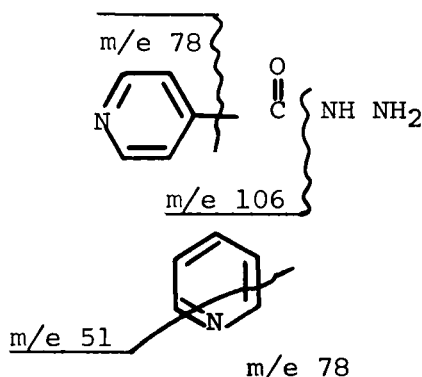
protons resonance by 0.1 ppm.

2.16 E.S.R. Spectrum

Hillerbrand and co-workers¹⁹ used Electron Spin Resonance to study charge transfer interactions between isoniazid and copper ions.

2.17 Mass Spectrometry

Gillis²¹ has discussed the fragmentation pattern for isoniazid and similar compounds. Figure 5 shows the electron-impact mass spectrum obtained on an AEI MS902 mass spectrometer equipped with a data acquisition system. The M^+ occurs at m/e 137 and the fragment ions result from either direct bond cleavage (m/e 106, 78) or through the elimination of HCN from the pyridyl ring (m/e 51).



2.2 Physical Properties of the Solid

2.21 Melting Characteristics

The melting point of isoniazid is used as specification in the United States Pharmacopoeia²³ and European Pharmacopoeia³. The melting point occurs between 170 and 174°C.

2.22 D.T.A. and D.S.C.

Differential thermal analysis was used to study isoniazid before the technique gained its current popularity^{24,25}. Pirisi²⁶ showed that isoniazid in the presence of zinc, copper and iron salts and mercuric oxide gives an abnormal D.T.A. pattern.

Dr. Jacobson²⁷ has shown that the

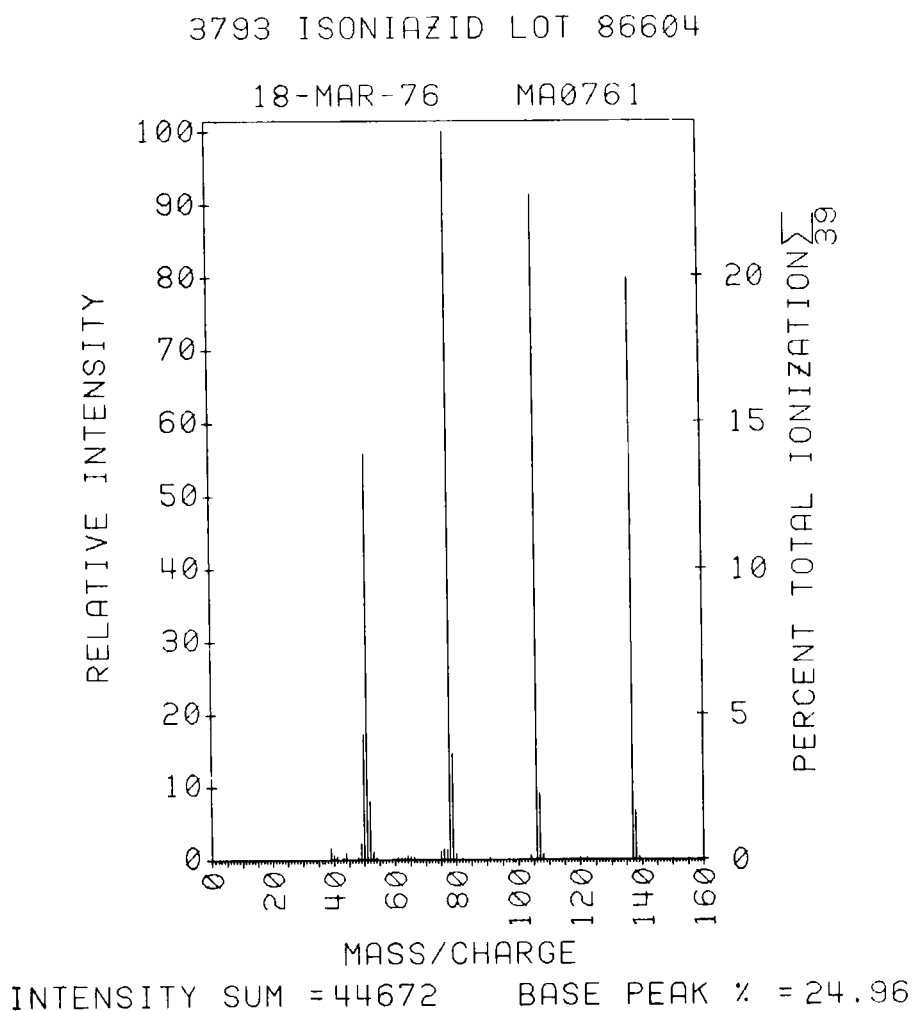


Figure 5: Low-resolution mass spectrum of isoniazid.

Squibb House Standard of isoniazid shows a sharp endotherm at 170°C using DuPont thermal analysis equipment.

The purity of this standard was determined to be 99.95 mole percent using a Perkin Elmer DSC-1B differential scanning calorimeter²⁷.

2.23 T.G.A.

Thermogravimetry can be used to determine moisture or residual solvents in isoniazid. When the Squibb House Standard was tested no loss on drying was recorded²⁷.

2.24 Electrical Moment

Lumbroso and Barassin²⁸ determined that the electrical moment of isoniazid was 2.92 μ .

2.25 Electrical Conductivity

The electrical conductivity of a compressed tablet of isoniazid was determined at temperatures between 50 and 150°C²⁹.

2.26 Crystal Characteristics

Bhat and co-workers³⁰ have reported that isoniazid crystals are orthorhombic, space group P 2₁2₁2₁, with a, 14.915 (15) b, 11.400 (10) c, 3.835 (5) Å, d (measured) = 1.417 (7) d (calculated) = 1.395 and Z = 4.

2.27 X-Ray Diffraction

The powder x-ray diffraction curve for isoniazid is shown in Figure 6³¹. The relative intensities for the various peaks are given below:

<u>Interplanar Distances</u> d (ANGSTROMS)	<u>Relative Intensities</u>
8.84	0.098
7.30	0.408
6.10	0.398
5.64	0.451
5.25	1.000
4.49	0.502
3.69	0.296
3.51	0.398
3.42	0.102
3.36	0.068
3.27	0.197
3.10	0.235
3.04	0.060
3.01	0.058
2.80	0.170
2.63	0.076
2.47	0.168
2.42	0.115
2.33	0.187

2.3 Solubility

2.31 Water Solubility³²

Fourteen grams of isoniazid are soluble in 100 ml of water at 25°C. Twenty-six grams are soluble in 100 ml of water at 40°C.

2.32 Solubility in Solvents^{32,33}

<u>Solvent</u>	<u>Solubility</u>
ethanol (25°C)	2 g/100 ml
ethanol (boiling)	10 g/100 ml
chloroform	0.1 g/100 ml
ethyl ether	very slightly soluble
benzene	insoluble

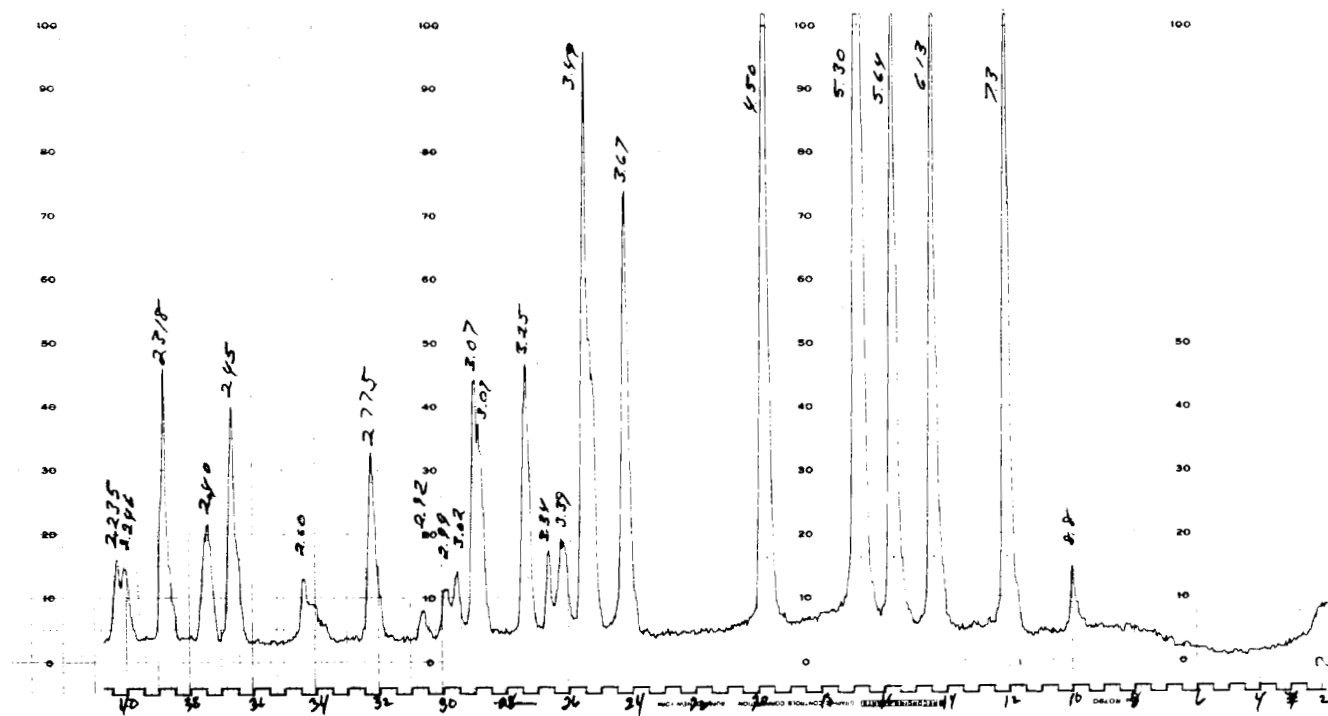


Figure 6: X-ray powder-diffraction pattern of isoniazid.

2.4 Physical Properties of Solution

2.41 pH

The pH of a solution (1 in 10) should be between 6.0 and 7.5²³.

2.42 Dissociation Constant

There is a discrepancy in the literature on the dissociation constants of isoniazid. This is in part due to the different methods of measurement employed.

Fallab³⁴ determined the basic dissociation constant as 3×10^{-11} measured conductometrically. Canić and Djordjević³⁵ established that the 1st basic constant should be ascribed to the pyridine nitrogen and the 2nd to the hydrazine group. This is contrary to previous work by Cingolani and Gaudiano³⁶.

Nagano and co-workers³⁷ determined the dissociation constants potentiometrically as $pK_1 = 2.13$, $pK_2 = 3.81$, $pK_3 = 11.03$.

Salvesen and Glendrange³⁸ determined the dissociation constants in 1.0M sodium chloride solution as $K_1 = 9.80 \times 10^{-3}$ and $K_2 = 1.42 \times 10^{-4}$.

Zommer and Szuszkiewicz¹¹ have established $pK_1 = 10.75$ and $pK_2 = 11.15$ and protonation constants of 3.57 for the pyridine N and 1.75 for the hydrazide N.

Rekker and Nauta⁶⁵ found that solutions of isoniazid became yellow at pH 10 and 2.7. The color is reversible on changing the pH. They explained this behavior on the basis of the existence of two positive ions, a monovalent yellow positive ion and a divalent colorless positive ion. The pK values are $pK' = 2.00$, $pK'' = 3.6$ and $pK''' = 10.8$.

2.43 Photolysis Constant

Salvesen and Eikill³⁹ established the photolysis constants for isoniazid at 20°C and 370 nm in M NaCl solution as $k_1 = 1.00 \times 10^{-2}$ and $k_2 = 1.45 \times 10^{-4}$.

2.44 Oxidation Potential

The oxidation potentials for isoniazid at various pH values were determined by Vulterin⁴⁰.

<u>Solution in</u>	<u>Ef</u>
1N HCl	0.78
0.025M Na ₂ B ₄ O ₇	0.25
3N NaOH	-0.22

3. Metal Complexes

Isoniazid forms metal complexes with many divalent ions. These complexes have been used in the determination of isoniazid (see Sections 6.22, 6.25 and 6.29).

Tamura and Nagano⁴¹ have determined the consecutive formation constants for the complex formed between isoniazid and Cd(II). The experiments were carried out at pH 7.2 (adjusted with NaOH) in M NaNO₃ using 0.001M Cd(NO₃)₂ at 25°C. The determination was made polarographically. The values determined were $k_1 = 35$, $k_2 = 0.57$, $k_3 = 52.5$. At high concentrations of isoniazid yellow crystals of Cd (INH)₂ (NO₃)₂·H₂O precipitated from solution indicating that contrary to the polarographic data that the 2:1 complex is more stable than the 3:1 complex. By pH titration the stepwise formation constants were $k_1 = 12.2$, $k_2 = 12.6$, and $k_3 = 3.4$.

The same authors⁴² studied the formation constants of isoniazid and Cu(II), Zn, Ni(II), Co(II) and Mn(II).

The complexes of copper and isoniazid have been extensively studied by Ishidate⁴³.

4. History, Synthesis and Manufacturing

Isoniazid was first prepared by Meyer and Mally⁵⁰⁰ in 1912 by heating a mixture of isonicotinic acid and hydrazine above 300°C. The activity of the compound against Mycobacterium sp. was first recognized by Chorine⁵⁰¹ and by Huant⁵⁰² in 1945. The drug was reported as a useful tuberculostatic agent by Farbenfabriken-Bayer, A.G., Hoffmann-LaRoche, Inc. and E. R. Squibb & Sons, Inc.

in 1952⁵⁰³.

The basic method of manufacture of isoniazid is the condensation of hydrazine with a γ -substituted pyridine.

Hydrazine can be directly condensed with isonicotinic acid. The water formed in the reaction is usually removed by azeotropic distillation^{44,45,46,47}.

Esters of isonicotinyl acid can be hydrolyzed and the resulting acid condensed with hydrazine. Ammonia is usually employed for the hydrolysis⁴⁸.

γ -Picoline can be oxidized in 70% sulfuric acid with manganous dioxide to form isonicotinic acid. The corresponding acid chloride is made with thionyl chloride. The acid chloride is then reacted with hydrazine in anhydrous benzene to yield isoniazid⁴⁹. In a modification of this procedure the acid chloride is reacted with ethanol to form the ethyl ester which is then reacted with hydrazine in ethanol to form isoniazid⁵⁰.

In a similar manner one can oxidize 2,4 dimethylpyridine with selenium and sulfuric acid.

The mixture is neutralized with ammonia. A mixture of isonicotinic acid, isonicotinamide and isonicotinic hydrazide is formed⁵¹.

5. Stability

The stability of isoniazid has been studied extensively in solution and in various pharmaceutical preparations. Of particular interest is the reaction of the hydrazine group with naturally occurring aldehydes and ketones such as sugars or ketoacids and the complexation of isoniazid with metal ions.

Lewin and Hirsch⁵² have shown that non-ionic chelating material can largely prevent the degradation of isoniazid when neutral and alkaline solutions are autoclaved. They noted that Cu(II) and Mn(II) ions accelerated the degradation of isoniazid in the presence of hydrogen peroxide.

Poole and Meyer⁵³ reported that isoniazid is unstable in human or rabbit plasma while it is

stable for several weeks in buffered aqueous solutions at pH values below 8. The instability in plasma is quite marked even at refrigerator temperatures.

Kakemi and co-workers⁵⁴ have studied the degradation of isoniazid in aqueous solution under anaerobic conditions. Alkaline hydrolysis under aerobic conditions yields a mixture of isonicotinic acid, isonicotinamide and 1,2 diisonicotinoyl hydrazine plus small amounts of unidentified products. Under anaerobic conditions isonicotinic acid and 1,2 diisonicotinoyl hydrazine were the principal products. When EDTA was added to the reaction mixture only isonicotinic acid was formed. First order kinetics were followed.

Inoue⁵⁵ found that at pH 3.1 under anaerobic conditions isoniazid hydrolyzes to form isonicotinic acid. Pseudo first order kinetics are followed. At lower pH values the effect of buffer type can be seen. Activation energies were calculated for the hydrolysis by different ionic species.

Horioka and co-workers⁵⁶ found that losses of isoniazid were encountered when the drug was blended with various antacid preparations. The effect of temperature, humidity and pH on the stability was determined.

Hald⁵⁷ found that isoniazid underwent slow oxidation in aqueous solution, but in the presence of sucrose the isoniazid reacted with the aldohexoses formed on inversion. The reaction with sucrose could be inhibited by the addition of 0.3% sodium citrate.

Pawelczyk and co-workers⁵⁸ found that as long as conditions were kept anaerobic that the decomposition of isoniazid in the pH range 3 to 7 followed first order kinetics. They reported that a 1% solution of the drug was 37 times more stable at pH 6 than at pH 3. The effect of different buffer species on the rate of the reaction was noted.

Wu and co-workers⁵⁹ investigated the browning reaction between lactose and isoniazid in the

solid state with diffuse reflectance spectrophotometry. Thin-layer chromatography was used to demonstrate the presence of isonicotinoyl hydrazones of lactose and hydroxymethylfurfural.

Inoue⁶⁰ has established the effect of the presence of copper (II) ions on the rate of oxidation of isoniazid in solution. The reaction products were isonicotinic acid, isonicotinamide, 1,2-diisonicotinoylhydrazine, isonicotine-carboxaldehyde and isonicotinoyl hydrazone. The copper chelates of isoniazid are degraded by a first order reaction and the rate is determined by the ratio of the concentration of chelated species present.

Inoue and Ono⁶¹ have established the kinetics of the degradation of isoniazid in the presence of Manganese (II). Shchukin⁶² has studied the reaction of copper (II) with isoniazid.

Kakemi and co-workers⁶³ studied the stability of the sodium methanesulfonate salt of isoniazid from pH 3 to 9.

Rao and co-workers⁶⁴ have demonstrated that isoniazid in syrup formulations undergoes hydrazone formation with the free glucose that is present. Absorption of this hydrazone is reported to be impaired. The authors suggest the use of sorbitol as a replacement for sucrose.

6. Analytical Chemistry

6.1 Identity Tests

A large number of identity tests have been established for isoniazid. Most of these are colorimetric and are reported below in tabular form.

<u>Reagent</u>	<u>Color</u>	<u>Reference</u>
p-Dimethylaminobenzaldehyde	intense yellow	66,67,68,74,85,86
Alkaline $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}$	intense orange	69,74
$\text{K}_4[\text{Fe}(\text{CN})_6]$ + light	pink	70
Dinitrochlorobenzene	purple	71,74,86
o-Dinitrobenzene	violet	72
Reduction with Zn/HCl and phenylhydrazine	yellow	73
1,2 Naphthoquinone-		
4-Sulfonic acid + NaOH	bright red	74,75,76
1,2,4-Aminonaphtholsulfonic acid	orange to red	77
SbCl_3 , SbCl_5 or AsCl_3	--	78
Epichlorohydrin	red	79
Dimethylglyoxime	red	80
Ethylenic dicarboxylic acids (fumaric, maleic acids, etc.)	yellow	81
Naphthoquinone- HgCl_2	--	82
3,5-Dinitrosalicylic acid	brown red	83
Ninhydrin	red orange	84,85
BrCN and NaOH	green-blue fluor.	85
Benzyl chloride NaOH	blue fluorescence	85
Dragendorff's Reagent	red	86,94

In addition to these color reactions a number of colored precipitates can be formed on the addition of metal salts or acids to isoniazid.

<u>Reagent</u>	<u>Color of Precipitate</u>	<u>Reference</u>
AgNO ₃	White	74, 94
SeO ₂	Red	78, 87, 88
HgCl ₂	White	74
CuSO ₄	Blue	86
Hg ₂ Cl ₂	White	86
KI	Amorphous mass	86, 93, 95
AuI	Dark crystals	86
Lead acetate + KI	Yellow acicular crystals	86
KBr	Effervescence followed by black and colorless crystals	86
P ₂ O ₅ ·I ₂ WO ₃	Precipitate	86
Picrotonic acid	Green-yellow	86, 93
Tannic acid	ppt	86
Vitali's reagent	Yellow mass	86
Mecke's reagent	Rose-sienna	86
Fröhde's reagent	Blue	86
Mandelin's reagent	Red	86
Alloxan	White ppt	89
Disulfimides	--	90
Methyliodide	--	91
K ₂ Cr ₂ O ₇	--	92
Phosphomolybdic acid	--	92
Picric Acid	Yellow needles	93, 94
Reinecke's salt	--	94
Styphnic acid	--	94

Kay's reagent	--	94
Vaillie's reagent	--	94
Na ₂ PtBr ₆	--	94

Feigl and co-workers⁹⁶ have reported a spot test in which isoniazid is quaternized and pyrolyzed with Na₂S₂O₃ at 180°C. Acidified Fe [Fe(CN)₆]⁷ is used for detection.

Popkov⁹⁷ and Amelink⁹⁸ have reported on microcrystalline techniques for the detection of isoniazid.

6.2 Methods of Analysis

6.21 General Reviews

Deltombe⁹⁹, Slouf¹⁰⁰, Robles and Unzueta¹⁰¹, Yalcindag¹⁰², Brandys¹⁰³ and Garcia and co-workers¹⁰⁴ have all published reviews on the qualitative and quantitative determination of isoniazid.

6.22 Colorimetric methods

A number of authors have formed hydrazones of isoniazid with various aldehydes and ketones and used the highly colored products to determine the drug. Of the various aldehydes used, p-dimethylaminobenzaldehyde appears to be the most popular^{105,106,107,108,109,110,111}. Benzaldehyde^{57,160,161} and vanillin^{113,197,198} have also been used. The official method of the AOAC is the reaction of isoniazid with benzaldehyde in sodium bicarbonate solution. The absorbance of the hydrazone is measured at 302 nm. The absorbance at 375 nm (background) is subtracted as a correction¹⁶⁹.

Sodium 1,2-naphthoquinone-4-sulfonate reacts with the hydrazide portion of isoniazid in alkaline solution to produce an orange-red color with a maximum at 480 nm^{114,115}. 2-3-Dichloro-1,4-naphthoquinone reacts

with isoniazid to give a blue color in alkaline solution^{116,117}. The reaction is useful with pharmaceutical preparations which also contain sodium aminosalicylate¹¹⁸. An assay utilizing 1, 4-naphthoquinone has also been reported¹¹⁹.

Isoniazid reduces phosphomolybdate in alkaline solution to molybdenum blue^{120,121}. In a similar reaction molybdophosphotungstate gives a blue color¹²². An assay utilizing molybdic acid in alkaline acetone solution has also been reported¹²³.

Isoniazid reacts with cyanogen chloride^{124,125,126}, chlororhodanamine^{127,128} or cyanogen bromide¹²⁹ to form glutaconic dialdehyde which can then be condensed with barbituric or 2-thiobarbituric acids to yield colored polymethine dyes.

Isoniazid reduces ferricyanide to ferrocyanide. The amount of ferrocyanide can be determined by the addition of ferric ion to yield a blue color^{130,131}.

Sodium pentacyanoaminoferroate reacts with isoniazid to give a yellow chromogen¹³².

Isoniazid reacts with 1-chloro-2, 4-dinitrobenzene in alkaline solution to give a purple color^{133,74,134}. 1-Fluoro-2,4 dinitrobenzene also reacts in a similar manner¹³⁵.

Isoniazid forms colored complexes with many metals which can be used in analytical methods. Complexes can be formed with ammonium vanadate^{136,137,230}, ferric chloride and 2,2' bi-pyridine¹³⁸, copper¹³⁹ and Nickel(II) and ferric ion¹⁴⁰.

Reinecke's salt forms a water insoluble precipitate with isoniazid. This precipitate dissolves in acetone and the concentration of isoniazid can be determined colorimetrically^{141,142}.

The following compounds have also been used in colorimetric assays for isoniazid.

<u>Reagent</u>	<u>Reference</u>
chloropicrin	504
epichlorohydrin	143
ninhydrin	144
triphenyltetrazolium chloride	145
9-chloroacridine	146
dinitrobenzoic acid	147
p-aminosalicylate-HVO ₃	148
1,2,4-aminonaphtholsulfonic acid	77
p-nitrophenyldiazonium fluoroborate	149
7-chloro-4 nitrobenzo-2-oxa-1,3-diazole	150
acid chrome dark blue	151
2-bromo-1-acetonaphthone	112
N-(4-pyridyl)pyridinium chloride	152
picryl chloride	174

6.23 Spectrophotometric Methods

A number of authors have utilized the strong absorbance of isoniazid in the ultraviolet as a means of determining the concentration of the drug. In many methods the authors take the spectrum both in alkaline and acid solution as an identity test^{153,154,155,156,157,158,159,165,237}. Isoniazid can be determined in the presence of p-aminosalicylate by an ultraviolet assay^{162,163,164}.

6.24 Fluorimetric Methods

Although isoniazid does not have any native fluorescence several sensitive fluorometric assays have been reported for the drug. Isoniazid is coupled with 2-hydroxy-1-naphthaldehyde to give a yellow-green fluorescence. The compound has an excitation maximum at 495 nm and an

emission maximum at 534 nm^{166,167}.

In another method the pyridine ring is cleaved with cyanogen bromide to form glutacondialdehyde. A Schiff's base is then formed with 4-aminobenzoic acid which has an excitation maximum at 336 nm¹⁶⁸.

6.25 Titrimetric Methods

A large variety of titrimetric methods have been employed for the determination of isoniazid in bulk and in formulated products.

A series of reviews have been written on titrimetric methods^{170,171,172,173,202}.

The official methods of analysis in the U.S.P.²³, B.P.¹⁷⁴ and European Pharmacopoeia³ are titrimetric methods.

In the U.S.P.²³ a nitrite titration is utilized. In the B.P.¹⁷⁴ the isoniazid is reacted with bromine and the excess bromine is titrated with thiosulfate after the liberation of iodine by the addition of potassium iodide. In the European Pharmacopoeia³ a direct titration with bromate is utilized with the addition of ethoxychysoidine as an indicator.

The various titrimetric methods are summarized in the Table.

<u>Reagent</u>	<u>Titrant</u>	<u>Indicator</u>	<u>Reference</u>
KBr, KBrO ₃ , KI	thiosulfate	starch	175, 177, 179, 182
KBr	KBrO ₃	ethoxychrysoidine	176, 106, 180, 184
Br ₂	alkali	phenolphthalein	178
-	KBrO ₃	methyl orange	181, 186
-	KBrO ₃	potentiometric	181, 183, 185, 187
I ₂	thiosulfate	starch	74, 188, 192, 196
-	KIO ₃	ethoxychrysoidine	189
KIO ₃ , KI	thiosulfate	starch	190, 193, 195
HI, K ₂ Cr ₂ O ₇ , KI	thiosulfate	starch	191
HClO ₄ ,	thiosulfate	starch	106, 194
ICl, KI	thiosulfate	starch	198
-	I ₂	thermometric	200
non-aqueous	HClO ₄	crystal violet or methyl violet	217, 211, 210, 209, 208, 207, 206, 204, 205, 216, 213, 201, 74, 57, 203, 214
-	NaNO ₂	-	233
non-aqueous	HClO ₄	Sb electrode	212, 276
non-aqueous	HClO ₄	glass electrode	215
-	NaClO ₄	potentiometric	218
non-aqueous	NaOMe	thymol blue	202
Cd ⁺⁺	Complexon III	eriochrome Black T	219, 221
Cd ⁺⁺	CaCl ₂	methylthymol Blue	220
Cu ⁺⁺ , NH ₄ SCN	AgNO ₃	-	222
Cu ⁺⁺ , NH ₄ SCN	EDTA	murexide	223, 224

HCl, $K_3Fe(CN)_6$	$ZnSO_4$	dead stop	225
$AgNO_3$	Complexon III	Eriochrome I	226
$AgNO_3, K_2[Ni(CN)_4]$	$MgSO_4$	Eriochrome Black T	227, 228
Complexon III			
-	$CO(OAc)_3$	Pt electrode	229
$CuSO_4$	Na versenate		230
Cu^{++}, NH_4SCN, KI	thiosulfate	starch	231
$AgNO_3$	NH_4SCN		232
-	$KMnO_4$	diphenylamine	234, 235, 236
-	$K_2Cr_2O_7$		235
-	$NaNO_2$	starch iodide paper	237
KBr	$NaNO_2$		238
Reinecke's salt,			
$AgNO_3$	NH_4SCN	Fe alum	239
Reinecke's salt,			
$AgNO_3$	NH_4SCN	Ag electrode	240
H_2SeO_3, KI	thiosulfate	starch	241, 242
$NH_4OH, AgNO_3$	NH_4SCN	Fe alum	243
Zn-Cu Couple, H_2SO_4	$NaOH$	methyl red	244, 245
Chloramine T, KI	thiosulfate	starch	246, 248
-	chloramine T	indigo carmine	247, 249
$NaVO_3$	$Fe(NH_4)_2(SO_4)_2$	N-phenylanthranilic acid	250, 254
Hg(II) EDTA	Pb^{++}	methylthymol blue	251
OsO_4	$NaVO_3$	diphenylamine	252, 253
H_3PO_4	NH_4VO_3	photometric	255

Nessler's reagent	sodium diethyl- dithiocarbamate	CuSO ₄	256
I ₂	hydrazine	starch	257
-	ammonium hexanitro- cerate(IV)	α-naphtho- flavone	258
Ce(SO ₄) ₂	Mohr's salt	Pt electrode	259
-	Ce(NO ₃) ₄	Pt electrode	260
K ₂ Cr ₂ O ₇	Mohr's salt	diphenylamine	261
isopropenyl tri- chloroacetate	NaOH	bromphenol blue	262
KOH	K ₃ Fe(CN) ₆	Pt electrode	263, 264
K ₃ Fe(CN) ₆ , KOH, H ₂ SO ₄ , KI	thiosulfate	starch	265

6.26 Electrochemical Methods

A number of authors have detailed polarographic methods for isoniazid. The reduction apparently occurs in two steps (total of 4 electrons) but the steps are not sufficiently well separated to be utilized analytically, so that the single wave is used. The half wave potential becomes more negative at higher pH values, but the height did not change greatly over the pH range studied^{266,153,267,268,269,270,271,272,273,274,275,277,278,279,280,281}.

A.C. Polarography has been used by Sato²⁸² and Vallon and co-workers²⁸³ in the assay of isoniazid. Okuda and co-workers²⁸⁴ reacted isoniazid with 1,2-naphthoquinone-4-sulfonic acid and have then used polarography to measure the reaction product.

Several authors have reported coulometric methods for the analysis of isoniazid with electrochemically generated chlorine^{285,286} or bromine^{286,287,288,289,290}.

6.27 Gravimetric Methods

Relatively few gravimetric assays have been reported for isoniazid. This is probably because of the large number of colorimetric, titrimetric and electrochemical methods available which are faster and more convenient than the gravimetric methods.

Leal and Alves²³⁴ have reported an assay using picric acid to form a water insoluble salt.

Akiyama and co-workers²⁹¹ precipitate isoniazide as the Cu(II) or Hg(II) salts. The salts are redissolved in hydrochloric acid and the metal is then reprecipitated as the sulfide which is determined gravimetrically.

The zinc²⁹² and cadmium²⁹³ salts can be measured by direct gravimetry. The benzylidene derivative can be determined either gravimetrically or volumetrically²⁹⁴. Isoniazid can be quaternized and the salt can be then measured volumetrically or gravimetrically²⁹⁵. The phosphotungstate of isoniazid can be determined gravimetrically¹⁶⁴.

6.28 Microbiological and Enzymatic Methods

Several agar diffusion microbiological assays utilizing strains of Mycobacterium have been reported for isoniazid^{296,297,298}.

Isoniazid inhibits many enzyme systems and a number of these might be selected as the basis of enzymatic assays. Examples of enzyme systems which are inhibited are pea cotyledon amine oxidase, carrot root L-glutamic decarboxylase and wheat seedling transaminase²⁹⁹. The inhibition is reversed by the presence of keto acids.

6.29 Miscellaneous Methods

Oscillometric titrations have been used to determine isoniazid^{300,301}. Isoniazid can be assayed gasometrically after oxidation with iodate³⁰² or ferricyanide^{303,304}.

Conductometric titrations with sodium hydroxide or hydrochloric acid have been

used to measure isoniazid content^{305,306}.

The copper chelate of isoniazid is soluble in methylisobutyl ketone. The copper content of the chelate is determined in the organic phase by atomic absorption spectrometry³⁰⁷.

Isoniazid in pure solutions can be determined by refractometry³⁰⁸.

6.3 Chromatographic Methods

6.31 Paper Chromatography

Numerous paper chromatographic systems have been used to separate isoniazid from intermediates used in the synthesis, degradation products and metabolic products. Since isoniazid absorbs strongly in the ultraviolet and gives a number of color reactions³⁰⁹ there is no problem in detecting or quantitating the drug after the separation has been completed. A table of some paper chromatographic systems is given below:

<u>Solvent System</u>	<u>Detection</u>	<u>Use</u>	<u>Ref.</u>
Water saturated butanol	Cl ¹⁴ labelled	Urine metabolites	310
Isoamyl alcohol-water-acetic acid(50:50:1.5)	CNBr, Microbiol.	Urine metabolites	311
Isopropanol-water(85:15)	--	Urine metabolites	312,313
Butanol-ammonia	--	Derivatives	314
1st Dimension sec.butanol-water (saturated)			
2nd Dimension isoamyl alcohol-acetone-acetic acid-water (56:24:6:14)	CNBr- o-phenyl-enediamine dimethyl-benzaldehyde	Urine quantitation	315

Butanol-10% NH ₄ OH(10:2) circular	Butanol sat.ammoniacal with silver nitrate	Impurities	316
Butanol-water(4:1) ascending	dimethylaminobenzaldehyde	Dosage forms	317
2,4-lutidine-isoamyl alcohol-water(5:100:9)	methanolic dinitro- chlorobenzene	Impurities	318
Butanol-HCl-pet. ether or Butanol-HCl-H ₂ O(paper sat. with KCl solution)	iodine-platinic iodide	other basic substances	319
(a)Butanol-ethanol-water (2:2:1)	ultraviolet	metabolites	320
(b)Butanol-pyridine-water (16:4:3)			
(c)Ethanol-1.5N NH ₄ OH-water (17:1:2)			
(d)Phenol-isopropanol-water (16:1:5)			
0.5 ammonium chloride	ultraviolet	metabolites	321
(a)Butanol saturated with water	--	metabolites in urine	322,323
(b)Propanol-water(80:20)			

(a) Isopropanol-25% NH_2OH (85:15)	} C^{14} and spray reagents	Metabolites	324
(b) Isopropanol-water (85:15)			
(c) Isopropanol-formic acid- water (80:10:10)			
Pyridine-Water (65:35)	} -- FeCl_3 and $\text{K}_3\text{Fe}(\text{CN})_6$	Metabolites	325
Isopropanol- NH_4OH -water (7:1:2)		Metabolites	326
Butanol-acetic acid-water (5:1:4)			
(a) Ethyl methyl ketone-acetone- formic acid water (40:2:1:6)	} {		327
(b) Ethyl methyl ketone-diethylamine- water (92:1:2:77)			
(c) Methyl isobutylketone-formic acid-water (ketone sat. with 4% formic acid)			
(d) Chloroform-methanol-formic acid- water (CHCl_3 sat. with 1 part H_2O and 1 part 4% formic acid)			
(e) Benzene-ethylmethyl ketone- formic acid-water (9 parts benzene plus 1 part ketone sat. with 2% formic acid)			
(f) Benzene-formic acid-water (benzene sat. with 2% formic acid)			

(a) Iso-propanol-water (17:3)	}	2,4,6 trinitro-	328
(b) Butanol-acetic acid-water (4:1:5)		benzene-sulfonic	
(c) 1.4M potassium phosphate buffer pH 7.0		acid	
Butanol-acetone-water (45:5:50)		chloranilic acid	329
Butanol-phosphoric acid-water (3:1:3)		--	330
(a) Butanol Sat. with water in atmosphere of NH ₃	}	copper sulfate	331
(b) 95% ethanol-M ammonium acetate (7:3) adjusted to pH 5		in ethanol then	
		0.1% benzidine in 50% aqueous ethanol	

6.32 Thin-layer Chromatography

In recent years several authors have developed thin-layer chromatographic system for isoniazid. These are presented in tabular form.

<u>System</u>	<u>Detection</u>	<u>Use</u>	<u>Ref</u>
Chloroform-methanol (8:2)	Folin-Ciocalteu	separation	332
Chloroform-acetone-diethylamine (5:4:1)	or Phospho-molybdate	from other drugs	
Cyclohexane-chloroform-diethylamine (4:5:1)			
Butanol-phosphoric acid-water (3:1:3)	--	derivatives	330
Acetone-methanol-NH ₄ OH (50:50:1)	dimethylamino-benzaldehyde	identification	333
(a) Methanol	}	identity	334
(b) Chloroform		test	
(c) Ethanol			
		5:1 mixture	
		10% CuSO ₄ and 10% NH ₄ OH	

Isopropanol-acetone (6:4)	--	separation of hydrazine	335
Chloroform-methanol (6:4)	--	separation of isonicotinic acid	335
Chloroform-methanol (125:60)	UV iodine	hydrazone with lactose	59
(a) Ethyl acetate-cyclohexane-dioxane-methanol-water-NH ₄ OH (50:50:10:10:1.5:0.5)	Ninhydrin or 0.5% H ₂ SO ₄	separation from drugs of abuse	336
(b) same solvent but (50:50:10:10:0.5:1.5)			
(c) Ethyl acetate-cyclohexane-NH ₄ OH-methanol-water (70:15:2:8:0.5)			
(d) Ethyl acetate-cyclohexane-NH ₄ OH-methanol (56:40:0.4:0.8)			
(e) same but (70:15:5:10)			
(f) Ethyl acetate-cyclohexane-NH ₄ OH (50:40:0.1)			
Methanol-NH ₄ OH-H ₂ O (100:1:4)	KMnO ₄ bromothymol other drugs blue		337

- | | | |
|---|---|---|
| (a) Chloroform-methanol-
13N ammonia (90:10:1)
(b) Benzene-methanol-
diethylamine (90:10:1)
(c) Chloroform-hexanol-
13N ammonia (90:10:0.2)
(d) Chloroform-ethyl acetate
13N ammonia (50:50:1)
(e) Chloroform-acetone-
acetic acid (90:10:1)
(f) Benzene-acetone-
diethylamine (50:50:1)
(g) Chloroform-acetone-
acetic acid (50:50:1) | } | 254 nm U.V. iron chloride-
hexacyanoferrate, molybdo-
phosphoric acid. Folin-
Ciocalteu, potassium
permanganate, ammoniacal
silver nitrate, amminepenta-
cyanoferrate, iodoplatinate,
iodine, Dragendorff, cinna-
maldehyde triphenyltetra-
zolium, dithiocarbamate
or ammonium molybdate |
|---|---|---|

338

Nishimoto and Toyoshima³³⁹ found that isoniazid showed tailing on thin-layer chromatography due to trace metals in the silica gel. When the adsorbent was treated with EDTA the tailing was eliminated.

Wijnne and co-workers³⁴⁰ found that isoniazid could be quantitated after thin-layer chromatography by coulometric titration. Schmidt³⁴¹ showed that isoniazid could be revealed on a thin-layer plate by exposure to iodine vapor. Kawale and co-workers³⁴² sprayed thin-layer plates with 1% mercurous nitrate to reveal isoniazid as black spots.

6.33 Ion exchange Chromatography

Tsuji and Sekiguchi³⁴³ have shown that isoniazid is quantitatively adsorbed on Dowex 50 cation exchange resin in various metal forms. The strength of adsorption decreases in the following order: $\text{Cu}^{++} \gg \text{Ni}^{++} \gg \text{Hg}^{++} \gg \text{H}^+ > \text{Co}^{++} > \text{Cd}^{++} \gg \text{Zn}^{++} > \text{Fe}^{++} > \text{Pb}^{++} > \text{Mn}^{++} > \text{Al}^{+++}$.

No adsorption occurs on resin in the Ba^{++} , Mg^{++} , Ca^{++} or Na^{+} forms.

Heller and co-workers³⁴⁴ separated acetylisoniazid from isoniazid on a column of Dowex 1-X8 in the pyruvate form.

Kakemi et al,³⁴⁵ developed a chromatographic method for the separation of isoniazid from some degradation products. Isoniazid is adsorbed on a weak cation exchanger such as Amberlite CG-50 in the hydrogen form. Isonicotinic acid is not adsorbed and is determined colorimetrically using cyanogen bromide. To determine isonicotinamide the sample solution is oxidized with alkaline ferricyanide and then passes through a column of strong anion exchanger such as Dowex 1-X8 in the chloride form. The amide is unchanged and is not adsorbed on the resin. Another degradation product, 1,2-diisonicotinoyl hydrazide is determined by adjusting the sample to pH 8.9 with borate buffer and determining the absorbance at 329 nm.

Peters and co-workers^{346,347} were able to separate and quantitate a large number of metabolites of isoniazid using Dowex AG-50-X4 resin in the hydrogen and ammonium forms. Selective color reactions were used to differentiate the groups of metabolites.

Fan and Wald³⁴⁸ separated p-aminosalicylic acid from isoniazid using a Dowex 2-X8 column. Inoue and co-workers³⁴⁹ used a system similar to that of Kakemi et al³⁴⁵ to separate isoniazid from its degradation products.

Lewandowski and Sybirska³⁵⁰ separated isoniazid from isonicotinic acid by paper chromatography using butanol saturated with water. The paper was connected with an ion exchange paper in the acid form. The spots were eluted with dioxane. The sharp zones on the ion exchange paper were visualized with picryl chloride.

Darawy and Mobarak³⁵¹ chromatographed several drugs on CM-82 carboxymethyl cellulose cation exchange paper using a water-

acetone-formamide(10:1:1) solvent system.

6.34 Other Chromatographic Methods

Barreto and Sabino³⁵² used a anhydrous sodium sulfate column eluted with chloroform-diethylamine(9:1) to concentrate metabolites of isoniazid from serum or urine.

Smolarek and Dlugosch³⁵³ separated isoniazid and p-aminosalicylic acid by paper electrophoresis in barbital buffer, pH 8.5. Barreto³⁵⁴ used two dimensional electrophoresis to separate the metabolites of isoniazid. Russell³⁵⁵ also used paper electrophoresis to separate several acyl hydrazides. A pH 2.0 acetate buffer was used.

Isoniazid was separated from several antituberculosis drugs by gas chromatography^{356,357}. A silanized chromosorb G coated with 6% QF1 was used. Gas chromatography was used to separate the products of oxidation of hydrazides with Fehling's solution³⁵⁸.

6.4 Determination of Isoniazid and its Metabolites in Body Fluids and Tissues

The methods described in this section were specifically developed for the determination of isoniazid in body fluids and tissues. Many of the methods are similar to other general analytical methods described in Section 6.2 perhaps differing only in the extraction procedure.

6.41 General Reviews

Terze and Dadiotou³⁵⁹ studied a number of color reactions to determine their application to blood level assays. Ginoulhiac³⁶⁰ also made a literature review of blood level methods. A critical review of methods for isoniazid determination has been written³⁶⁴.

6.42 Colorimetric Methods

Colorimetric methods are most popular for the determination of isoniazid in biological samples. The methods are listed in tabular form.

<u>Reagent</u>	<u>Pretreatment of sample</u>	<u>Type of specimen</u>	<u>Ref.</u>
Dimethylaminobenzaldehyde	acid hydrolysis	serum & urine	361,370, 375
Dimethylaminobenzaldehyde	none	urine	362,363
Dimethylaminobenzaldehyde	extraction into isoamyl alcohol- ether from alkaline solution	plasma and urine	365,366, 367,368, 369.
Dimethylaminobenzaldehyde	deproteinization with HClO ₄	serum	371
Dimethylaminobenzaldehyde	deproteinization with trichloroacetic acid	serum and tissues	372
Vanillin	none	serum	373,376, 377
Vanillin	deproteinization with trichloroacetic acid	serum	374,432, 433,434
Vanillin	extraction with organic solvent	serum	375
Vanillin	extraction with propanol	milk	378
Cinnamaldehyde	deproteinization with trichloroacetic acid	serum	379,380, 429,430
Cinnamaldehyde	extraction with butanol-chloroform	serum	381

o-Nitrobenzaldehyde	deproteinization with	serum	382
Salicylaldehyde-FeCl ₃	trichloroacetic acid		
	extraction into isoamyl	serum	383
	alcohol-ether from		
Salicylaldehyde	alkaline solution		
	none	biological	384
		fluids	
Salicylaldehyde	extraction with	cadavers	385
	acetone		
Glutaconic aldehyde	deproteinization with	plasma	386
	trichloroacetic acid		
β-diketone	none	biological	387
		materials	
Catechol	deproteinization with	citrate	388
	trichloroacetic acid	blood	
Catechol	automated method	serum	389
H ₂ O ₂ -CNBr	deproteinized	serum &	390
	serum	urine	
CNBr	deproteinized tri-	biological	391,404,
	chloroacetic acid	fluids	411,412
Alkaline hydrolysis-	deproteinized tri-	urine	392
CNBr	chloroacetic acid		
NH ₄ VO ₃ -H ₂ SO ₄	acid hydrolysis	urine	393,394,395,
			396,397,398,
			399,370,435
KCN, Chloramine T- barbituric acid }	--	plasma,urine	400,401
		tissues,serum	

1-amino-2-naphthol-4-sulfonic acid	--	urine biol. fluids	402
Naphthoquinone-4-sulfonic acid	--	urine	403,404,405,406,407
Naphthoquinone-4-sulfonic acid	deproteinization Zn(OH)_2	urine	408
2,4,6-trinitrobenzene-sulfonic acid	extraction methyl isobutyl ketone	whole blood	409,410
Dinitrochlorobenzene	--	urine	411
Dinitrochlorobenzene	deproteinized serum	serum	412,413
$\text{K}_3\text{Fe(CN)}_6$	--	serum	414
Sodium pentacyanoaminoferroate	deproteinized tissue	tissue, urine	415,416,417
$\text{K}_3\text{Fe(CN)}_6$	deproteinized with sodium tungstate	spinal fluid	418
Nitropentacyanoferroate	deproteinized with phosphoric acid	serum	419
Naphthoquinone	--	spinal fluid	420
Naphthoquinone	--	urine	
H_2O_2 , CNBr, aniline	trichloroacetic acid	blood	421
Picryl chloride	tungstic acid	blood	422
	extraction BuOH , Et_2O	urine	
KMnO_4 , BrCN, NH_3	protein-free filtrate	plasma	423,424
		spinal fluid	
		plasma	425

4-pyridylpyridinium	trichloroacetic acid	plasma	426
dichloride, NaOH, HCl	filtrate		
KBrO ₃ + methyl orange	acid tungstate	blood	427
Zn powder + heat	--	urine	428

6.43 Turbidimetric Method

Isoniazid reduces K_2HgI_4 to form HgI which is insoluble. The resulting turbidimetry can be measured to determine the amount of isoniazid present. Wagner and co-workers⁴³¹ have applied this method to blood following deproteinization with barium hydroxide and zinc sulfate.

6.44 Fluorimetric Methods

A number of fluorimetric methods for isoniazid have been reported. Hedrick and co-workers⁴³⁶ absorbed a protein free filtrate of serum on pH 6.5 Amberlite XE-64 ion exchange resin. The isoniazid was eluted with dilute acid and then reacted with hydrogen peroxide in pH 8.7 buffer. The oxidation product fluoresces at 415 nm when activated by ultraviolet light at 320 nm. As little as 0.05 γ /ml of serum can be determined.

Scott and Wright⁴³⁷ reacted salicylaldehyde with isoniazid and reduced the resulting hydrazone. The resulting compound was highly fluorescent. Reiss, Morse and Putsch⁴³⁸ assayed isoniazid fluorimetrically after absorption and elution from ion exchange resin and treatment with alkaline cyanogen bromide. Wilson, Lever and Small⁴³⁹ utilized the fluorescence of the zinc chelate of the hydrazone of isoniazid with pentane-2,4-dione in an assay in serum.

Ellard, Gammon and Wallace⁴⁴⁰ have developed specific fluorimetric assays for isoniazid, acetylisoniazid, mono- and diacetylhydrazine, isonicotinic acid and isonicotinylglycine in serum and urine. Boxenbaum and Riegelman⁴⁴¹ have also developed assays for isoniazid and its metabolites in whole blood.

Miceli, Olson and Weber⁴⁴² have established a micro method for

the fluorimetric determination of isoniazid in serum. As little as 25 μ l of serum can be used in the assay.

Peters, Morse and Schmidt⁴⁴³ and O'Barr, Keith and Blair⁴⁴⁴ have compared fluorimetric and microbiological assays for isoniazid in serum.

6.45 Electrochemical Methods

Lauermann and Otto⁴⁴⁵ hydrolyzed isonicotinic acid hydrazide and its metabolites to isonicotinic acid with alkali. The hydrolysis product was determined polarographically. The authors found that the results obtained by this method in the analysis of cadaveric fractions was comparable to those obtained when the method of Nielsch and Gießer⁴⁰¹ was used. The polarographic method was less time consuming.

Kane⁴⁴⁶ determined isoniazid in biological fluids without prior separation.

6.46 Gasometric Methods

The hydrazine group in isoniazid can be readily decomposed into nitrogen gas. Several authors have utilized this relatively selective finish for blood and urine level assays.

Strickland and Hentel⁴⁴⁷ reacted isoniazid with sodium iodate in alkaline solution. The assay is not effected by the presence of p-aminosalicylic acid which is often given in conjunction with isoniazid. Harting and Gerzanits⁴⁴⁸ used alkaline ferricyanide to liberate the nitrogen gas.

Ito and co-workers^{449,450} were able to selectively use copper, iron and chromium azometry to determine isoniazid and its various metabolites in urine.

6.47 Miscellaneous Chemical Assays

Verrotti and Bardelli⁴⁵¹ determined isonizid in cerebrospinal fluid by iodometric titration.

Schwenk and co-workers⁴⁵² employed a radioimmunoassay for the determination of isoniazid in biological fluids.

6.48 Microbiological Assays

Although isoniazid is readily measured in biological fluids and tissues by chemical assays, as with many antibacterial substances a number of microbiological assays for this substance have been proposed.

<u>Microorganism</u>	<u>Type of assay</u>	<u>Sensitivity</u>	<u>Ref.</u>
<u>Mycobacterium phlei</u>	agar diffusion	2.5-30 γ /ml	453
Koch bacilli	turbidimetric	--	454
tubercle bacteria	cord formation	--	455
bacilli	vertical diffusion	--	456
<u>Mycobacterium</u>			
<u>tuberculosis</u> HV37	vertical diffusion	--	457
<u>Mycobacterium</u>	agar diffusion	--	458
<u>tuberculosis</u>			
<u>Mycobacterium</u>	vertical diffusion	> 0.49 γ /ml	459
<u>tuberculosis</u>			
H ₃₇ Rv and H ₃₇ Ra BGG	assay of isoniazid in milk-agar diffusion		378
--	vertical diffusion		460
<u>Mycobacterium</u>	vertical diffusion		461
<u>tuberculosis</u>			
--	vertical diffusion		462
--	tube dilution		
--	vertical diffusion for urine		463,464

Bartmann and Freise⁴⁶⁵ studied the tissue binding of isoniazid with the microbiological assay. They found 40% binding with human tissue while mice and guinea pig tissue gave 80% binding. Incubating the tissue at an elevated temperature did not raise the recovery.

Nishi⁴⁶⁶, Poole and Meyer⁴⁶⁷, Tansini and co-workers³⁷⁶, Peters and co-workers⁴³³ and O'Barr *et al*⁴⁴⁴, all compared various chemical assays and microbiological assays. All workers conclude that the two methods gave comparable results.

6.49 Chromatographic Assays

The metabolism of isoniazid is complex and many workers have selected chromatographic assays to measure the drug in tissue and biological fluids. These methods provide the specificity that are not given by many chemical methods.

A large number of chromatographic systems are given in section 6.3. Many of these methods could probably be used to measure isoniazid in tissues and biological fluids. The methods given in this section have been developed just for this purpose.

Makino and co-workers⁴⁶⁸ followed the metabolism of isoniazid in liver and in urine by paper chromatography (water saturated butanol, 1% ammonia-isopropanol(3:20), butanol saturated with 0.02M phosphate buffer pH 7.4, 1% ammonia saturated butanol and butanol-acetic acid-water (4:1:5)).

Leuschner⁴⁶⁹ used sec-butanol saturated with water and isoamyl alcohol as developing solvents.

Iwainsky³¹³ separated the hydrazones of isoniazid and pyruvic and α -keto-glutaric acid from isoniazid with paper chromatography.

Sezaki⁴⁷⁰ separated isoniazid from pyrazinamide in urine by means of Amberlite IRA-400. Belles and Littleman⁴⁷¹ used Dowex 50-X8 to separate isoniazid from acetylisoniazid. Abiko

and co-workers⁴⁷² use Dowex 1-X10 to separate these as well as the hydrazone of glucuronic acid.

Peters, Miller and Brown³⁴⁶ utilized ion exclusion chromatography to separate metabolites of isoniazid into ionized, slightly ionized and unionized groups of compounds. The individual metabolites were measured with specific colorimetric assays.

Okudaira and co-workers⁴⁷³ used Dowex 1 and Dowex 50 columns in tandem to separate the various metabolites of isoniazid.

Paper chromatographic systems have been used to isolate the various metabolites of isoniazid^{474,475,352}.

Barreto and Sabino⁴⁷⁶ have described a two dimension separation of isoniazid metabolites using paper chromatography and paper electrophoresis. The same authors³⁵² have also used a sodium sulfate column developed with chloroform-diethylamine(90:10) to separate the metabolites of isoniazid.

Fartushnyi and Sukhin⁴⁷⁷ have used TLC to determine isoniazid and other drugs in cadavers.

Cattaneo, Fantoli and Ferrari⁴⁷⁸ claim that their chromatographic studies indicate that the tumorigenic effect of isoniazid in mouse lung is due to the large amount of isonicotinic acid produced in that organ.

Hughes⁴⁷⁹ separated acetylisoniazid from isoniazid by counter-current distribution (butanol-ethylene dichloride- 9:1 - 2M phosphate buffer pH 5.1).

Ozawa and Kiyomoto⁴⁸⁰ isolated three conjugated metabolites of isoniazid by paper chromatography. Cuthbertson et al⁴⁸¹ used paper chromatography to determine isonicotinoylglycine. They used the following systems:

Water saturated butanol

Methylethylketone:acetic acid:water(49:1:50)

Propanol:water(4:1)

Zamboni and Defranceschi⁴⁸² used a isopropanol:water(85:15) system to separate the hydrazones of pyruvic and α -ketoglutaric acid from

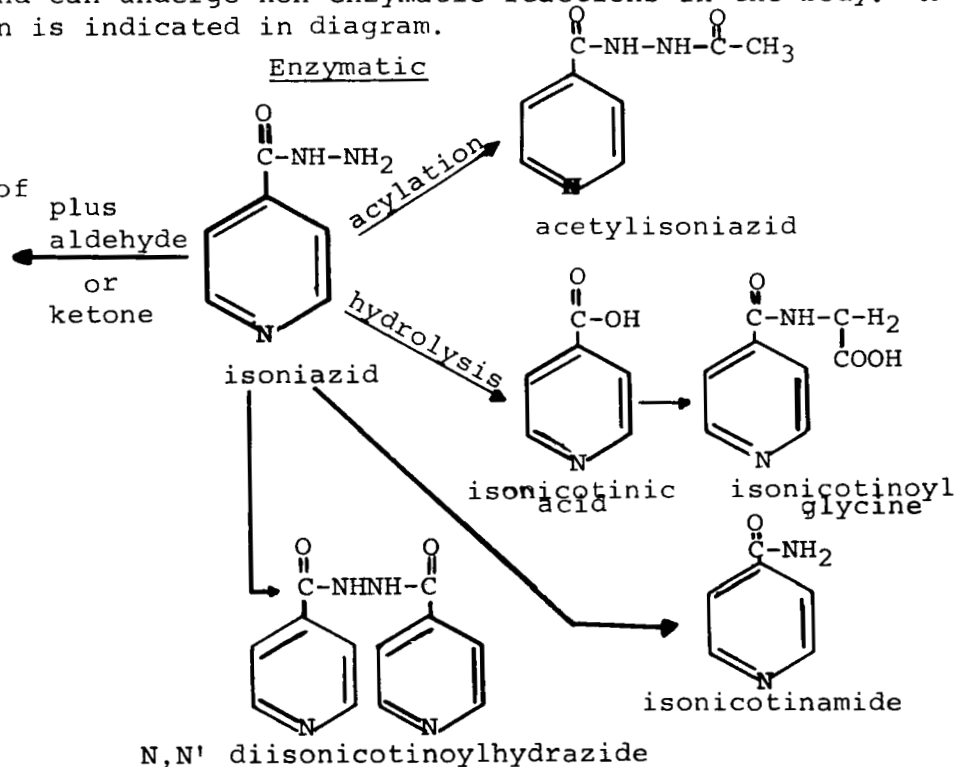
isoniazid.

7. Drug Metabolism

The drug metabolism of isoniazid is unusually complicated in that it is a very reactive molecule and can undergo non-enzymatic reactions in the body. A general metabolic pattern is indicated in diagram.

Non-enzymatic

isonicotinoylhydrazones of
glucose,
 α -ketoglutaric acid,
pyruvic acid etc.



The major metabolite of isoniazid is N-acetylisoniazid. The rate of acetylation is genetically controlled^{483,484 485}. It has been established that the slow acetylation is a autosomal recessive trait. The acylation occurs by N-acetyl transferase. Six hours after the oral administration of 4 mg/Kg of isoniazid fast acetylators have plasma concentrations of 0.2 µg/ml or less while slow acetylators have plasma levels higher than 0.4 µg/ml⁴⁸⁶.

In a metabolic scheme, such as the one indicated earlier, relative amounts of the various metabolites found in the urine will differ for each individual and will depend on genetic factors, previous drug history (enzyme induction) and general nutrition (availability of ketoacids).

Reviews on the drug metabolism of isoniazid have been prepared by a number of authors^{487,488, 489,490,491,492,493,494,495,496,497}.

Toth and Shimizu have reported that the continuous administration of N-acetylisoniazid in rats has markedly increased the incidence of lung tumors in this species. Since the N-acetyl derivative is a major metabolite in man this poses some questions on the long term administration of the compound⁴⁹⁷.

8. Biopharmaceutics

Kakemi and co-workers⁴⁹⁸ determined the rate of absorption of derivatives of isoniazid in the stomach and intestine. The authors report a rough correlation between degree of absorption and lipid-water partition coefficient.

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KANAMYCIN SULFATE

Paul J. Claes, Maurice Dubost and Hubert Vanderhaeghe

TABLE OF CONTENTS

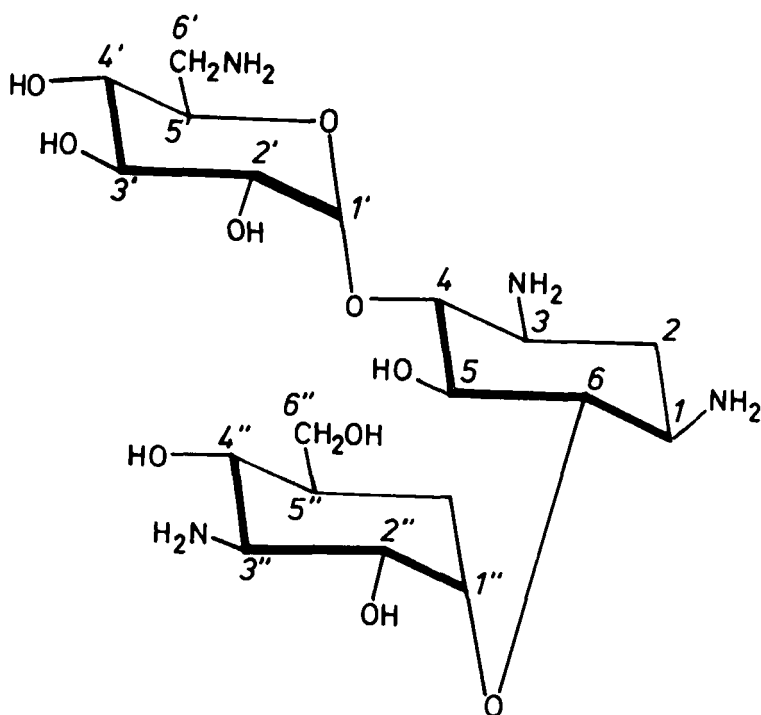
1. Description
 - 1.1. Name, Formula, Molecular Weight
 - 1.2. Appéarance, Color, Odour
 - 1.3. Definition of International Unit
2. Physical Properties
 - 2.1. Spectra
 - 2.11. Infrared Spectra
 - 2.12. Ultraviolet Spectra
 - 2.13. Nuclear Magnetic Resonance Spectra
 - 2.14. Mass Spectra
 - 2.2. Optical Rotation
 - 2.3. Electrometric Titration Curve-pK Values
 - 2.4. Crystal Properties
 - 2.5. Melting Range
 - 2.6. Thermal Analysis
 - 2.7. Solubility
3. Synthesis
 - 3.1. Fermentation-Biosynthesis
 - 3.2. Chemical Synthesis
4. Stability-Degradation
5. Inactivation by Enzymes
6. Mode of Action
7. Pharmacokinetics
8. Methods of Analysis
 - 8.1. Identification
 - 8.2. Determination of Sulfate
 - 8.3. Loss on Drying
 - 8.4. Microbiological Assay
 - 8.5. Assay of Kanamycin B

- 8.6. Chromatographic Analysis
 - 8.61. Paper
 - 8.62. Thin Layer
 - 8.63. Ion Exchange
 - 8.64. Gas Liquid
- 8.7. Electrophoretic Analysis
- 9. Determination in Body Fluids and Tissues
- 10. References Cited

1. Description

1.1. Name, Formula, Molecular Weight

Kanamycin or kanamycin A (I) is the major component of the antibiotic complex produced by certain strains of Streptomyces kanamyceticus¹. Its structure was established as O-(6-amino-6-deoxy- α -D-glucopyranosyl)-(1 \rightarrow 4)-O-[3-amino-3-deoxy- α -D-glucopyranosyl-(1 \rightarrow 6)]-1,3-diamino-1,2,3-trideoxy-scyllo-inositol. Since 1972 the compound has been listed in Chemical Abstracts under the heading D-streptamine, O-3-amino-3-deoxy- α -D-glucopyranosyl-(1 \rightarrow 6)-O-[6-amino-6-deoxy- α -D-glucopyranosyl-(1 \rightarrow 4)]-2-deoxy-. The numbering is given in the formula below. The carbon atoms of the 2-deoxy-



Kanamycin A free base : $C_{18}H_{36}N_4O_{11}$

M.W. 484.50

Kanamycin A monosulfate monohydrate :

$C_{18}H_{36}N_4O_{11} \cdot H_2SO_4 \cdot H_2O$

M.W. 600.59

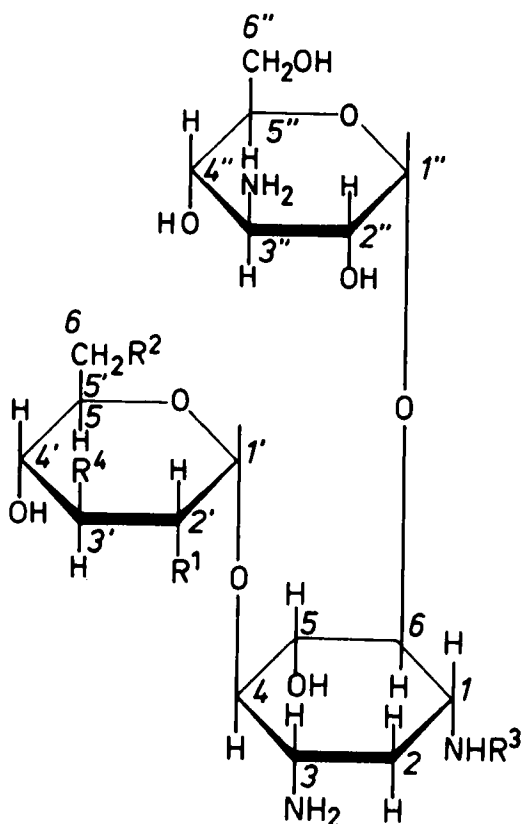
streptamine ring are numbered as 1,2,3,..., those of the amino sugar moieties linked at C-4 and C-6 of 2-deoxystreptamine respectively as 1',2',3',... and 1'',2'',3'',...

Kanamycin A is supplied in two forms, a crystalline monosulfate monohydrate and a salt with a higher sulfate content. The latter is more readily soluble in water and is designated in the Brit. Ph. Add. 1975² as kanamycin acid sulfate. The sulfate (SO_4) content calculated for the monosulfate monohydrate is 15.99 %. In most commercial samples the sulfate content varies from 16 to 16.4 %^{3,4}. The monosulfate monohydrate is reported in the U.S. Ph. XIX⁵ and in the Brit. Ph. 1973⁶ under the heading kanamycin sulfate. To avoid confusion the designation kanamycin monosulfate should be preferred. The limits of the pH (1 % aqueous solution) given in the Code of Federal Regulations⁷ and in the Eur. Ph.⁸ are from 6.5 to 8.5.

Kanamycin acid sulfate, the name used in Brit. Ph. Add. 1975, which is sometimes referred to as kanamycin bisulfate, is obtained by adding sulfuric acid to a solution of the monosulfate and drying by a suitable procedure. Its sulfate content (dry basis) may vary from 24 to 26 %. Percentages sulfate calculated for $\text{C}_{18}\text{H}_{36}\text{N}_4\text{O}_{11} \cdot 1.6 \text{H}_2\text{SO}_4$ and $\text{C}_{18}\text{H}_{36}\text{N}_4\text{O}_{11} \cdot 1.8 \text{H}_2\text{SO}_4$ are respectively 23.95 and 26.14 %. It is obvious from these figures, that the name kanamycin bisulfate is not a correct designation. The limits of pH given by the Eur. Ph.⁸ are from 5.5 to 7.5.

Kanamycin B (II) and kanamycin C (III) are two minor components of the antibiotic complex. They differ from kanamycin A in the nature of the amino sugar linked to the 4-position of the 2-deoxystreptamine moiety (2,6-diamino-2,6-dideoxy-D-glucose for II and 2-amino-2-deoxy-D-glucose

for III). Kanamycin B, also referred to as bekanamycin, is available as its sulfate salt under the name Kanendomycin^R (Meiji).



II kanamycin B : $R^1 = R^2 = \text{NH}_2$, $R^3 = \text{H}$, $R^4 = \text{OH}$

III kanamycin C : $R^1 = \text{NH}_2$, $R^2 = \text{OH}$, $R^3 = \text{H}$, $R^4 = \text{OH}$

IV amikacin : $R^1 = \text{H}$, $R^2 = \text{NH}_2$, $R^3 = \text{L}(-)\text{-CO-CH-}(\text{CH}_2)_2\text{-NH}_2$,
 $R^4 = \text{OH}$ OH

V tobramycin : $R^1 = R^2 = \text{NH}_2$, $R^3 = \text{H}$, $R^4 = \text{H}$

The two antibiotics amikacin (or BB-8) (IV) and tobramycin (V) are structurally related to the kanamycins. The former is obtained by selective N-acylation of kanamycin A at the 1-amino group with L(-) γ -amino- α -hydroxybutyric acid⁹. The latter is a 3'-deoxykanamycin B produced by Streptomyces tenebrarius¹⁰.

1.2. Appearance, Color, Odour

The monosulfate monohydrate is a white or almost white, odourless or almost odourless crystalline powder. The acid sulfate is amorphous.

1.3. Definition of International Unit¹¹

The International Reference Preparation is a sample of kanamycin monosulfate (17.2 % SO₄) established in 1959. The International Unit was defined in 1962 as the activity contained in 0.001231 mg of the International Reference Preparation, corresponding to a potency of 812 U/mg.

2. Physical Properties

2.1. Spectra

2.1.1. Infrared Spectra

Infrared spectra of kanamycin monosulfate monohydrate and of the free base have been published by Maeda¹². These spectra are typical for polyhydroxy polyamino compounds. However, no characteristic bands, which would permit differentiation from related aminoglycosidic antibiotics, are present.

2.12. Ultraviolet Spectrum

Kanamycin free base and its sulfate salts show end absorption only¹².

2.13. Nuclear Magnetic Resonance Spectra

The PMR spectrum of kanamycin free base, determined on a Varian XL-100 instrument at ambient temperature, is presented in the figure 1. The spectrum was obtained by dissolving 60 mg crystalline free base in 0.5 ml D₂O, containing sodium 3-(trimethylsilyl)propane-1-sulfonate as internal standard. In the spectrum, which is in agreement with that published¹³, signals appear in three separate regions. The lowest field contains two one-proton doublets, due to anomeric protons. The highest field shows two one-proton signals, due to the methylene group of the deoxystreptamine moiety. Signals from the remaining protons, attached to carbon atoms

Table I. PMR Spectral Assignments of Kanamycin A free base

<u>Assignment</u>	<u>Chemical Shift</u> [*]	<u>Coupling Constant</u>
2-H _a	1.22 (m)	J _{gem} = 13 Hz, J _{aa} = 12 Hz
2-H _e	1.96 (m)	J _{gem} = 13 Hz, J _{ea} = 4 Hz
3''-H	2.90 (m)	
2''-H	3.48 (m)	J = 3.8 Hz, J = 10 Hz
2'-H	3.55 (m)	J = 3.3 Hz, J = 9.5 Hz
3'-H	3.77 (m)	
1''-H	5.03 (d)	J = 3.8 Hz
1'-H	5.79 (d)	J = 3.3 Hz

d = doublet; m = multiplet; gem = geminal; a = axial; e = equatorial.

* The δ -values, given in this table, refer to sodium 3-(trimethylsilyl)propane-1-sulfonate as internal standard. They can be converted into δ -values, referring to TMS, by adding 0.48 ppm.

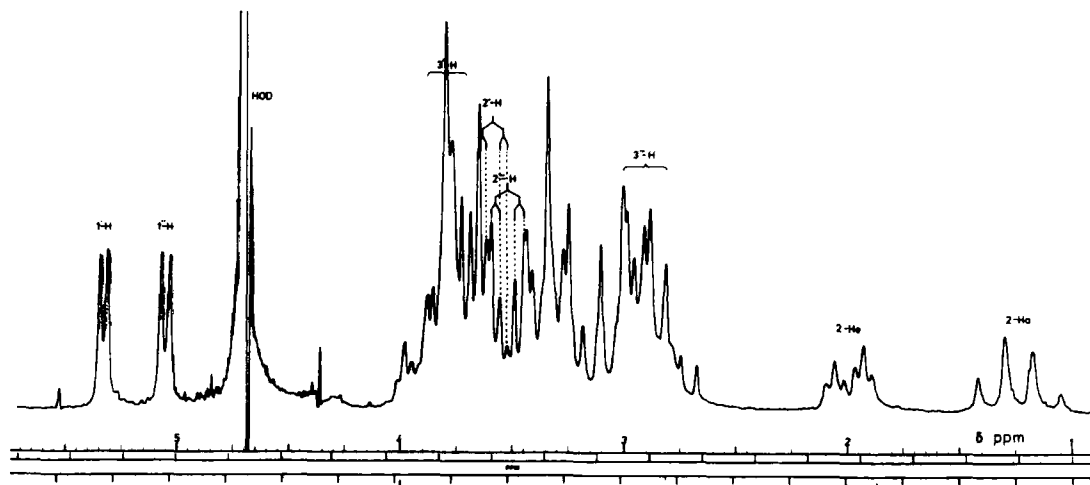


Fig. 1. PMR spectrum (100 Mc) of kanamycin A free base, taken in D_2O with sodium 3-(trimethylsilyl)propane-1-sulfonate as internal standard.²

bearing -NH_2 , -OH or -O- , are found in the central region. The spectral assignments, given in the figure and summarized in Table I, have been discussed in detail by Naganawa *et al.*¹³.

Spectral data, observed for a solution of the monosulfate of kanamycin A in D_2O solution and for its tetrahydrochloride, are given in Table II. It can be seen that protonation of the amino groups causes a downfield shift of some of the protons. This effect is less pronounced for the monosulfate.

The PMR spectrum of kanamycin B has been reported by Koch *et al.*¹⁵. Assignment of the signals in carbon-13 NMR spectra of kanamycin A¹⁶ and B¹⁷ have been reported recently.

Table II. Chemical Shift Values* observed for Kanamycin A Salts¹⁴

Protons	Kanamycin monosulfate**	Kanamycin 4 DCI***
2-H _a	1.48 (m)	1.98 (m)
2-H _e	2.16 (m)	2.6 (m)
CHO, CH ₂ O	2.95 - 4.2 (m)	2.95 - 4.2 (m)
CHN, CH ₂ N		
anomeric protons	5.09 (d) and 5.52 (d)	5.18 (d) and 5.58 (d)

* δ -Values relative to sodium 3-(trimethylsilyl)propane-1-sulfonate as internal standard.

** Saturated solution in D_2O

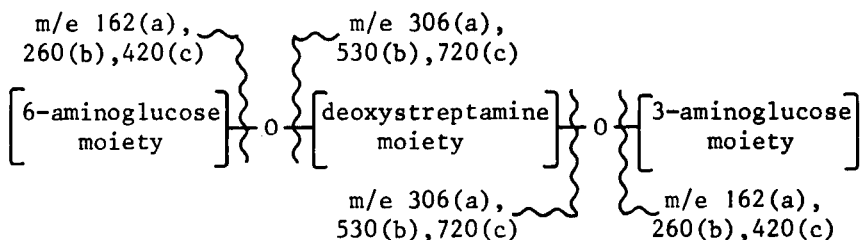
*** 35 mg kanamycin A free base in 0.35 ml 1N DCI.

2.14. Mass Spectra

The high- and low resolution mass spectra of volatile derivatives of kanamycin A (N-acetyl-N,O-methyl- and N-acetyl-O-trimethylsilylkanamycin) have been determined and interpreted by De Jongh *et al.*¹⁸. The electron impact spectra of both

derivatives show a very small molecular ion peak, which may be obscured by background or noise. More intense are the (M+1) peak in the spectrum of the N-acetyl-N,O-methyl derivative and the (M-15) peak in that of the N-acetyl-O-trimethylsilyl derivative. Other diagnostic peaks, observed in the spectra of both derivatives, result from a cleavage of glycosidic bonds or C-O bonds connecting a hexose to the deoxystreptamine unit. The m/e values of these peaks reveal the sequential arrangement and the gross structure of the saccharide- or the aminocyclitol units, of which kanamycin is composed. Mass spectra of deuterated analogs and the chemical ionization mass spectrum of N-acetyl-N,O-methylkanamycin A are also described in the paper of De Jongh *et al.*¹⁸.

Mass spectra of the underivatized free bases of kanamycin A and B and of other aminoglycoside antibiotics (up to the pseudotrisaccharide level) have been reported by Daniels *et al.*^{19,20}. The electron impact spectrum of underivatized kanamycin A shows a MH^+ peak at the highest mass ion. Other diagnostic fragment ions arise from glycosidic cleavage and from a cleavage of one of the sugar units. Some of the diagnostic peaks observed in the spectra of derivatized and underivatized kanamycin A are given in the following scheme :



- (a) Kanamycin A underivatized : $m/e\ 485\ (M+1)^+$
 (b) N-Acetyl-N,O-methylkanamycin A : $m/e\ 807\ (M+1)^+$,
 $m/e\ 806\ (M)^+$
 (c) N-Acetyl-O-trimethylsilylkanamycin A : $m/e\ 1156\ (M)^+$,
 $m/e\ 1141\ (M-15)^+$

2.2. Optical Rotation

The following specific rotations have been reported;

for the free base

$[\alpha]_D^{13} + 140^\circ$ (c 1, H_2O), $M_D + 67.830$	Maeda ¹²
$[\alpha]_D^{24} + 146^\circ$ (c 1, 0.1N H_2SO_4), $M_D + 70.737$	Cron <i>et al.</i> ²¹
$[\alpha]_D^{25} + 150.5^\circ$ (c 1, 0.2N H_2SO_4), $M_D + 72.917$	Claes <i>et al.</i> ²²

for the monosulfate

$[\alpha]_D^{13} + 121^\circ$ (c 1, H_2O), M_D 72.672	Maeda ¹²
--	---------------------

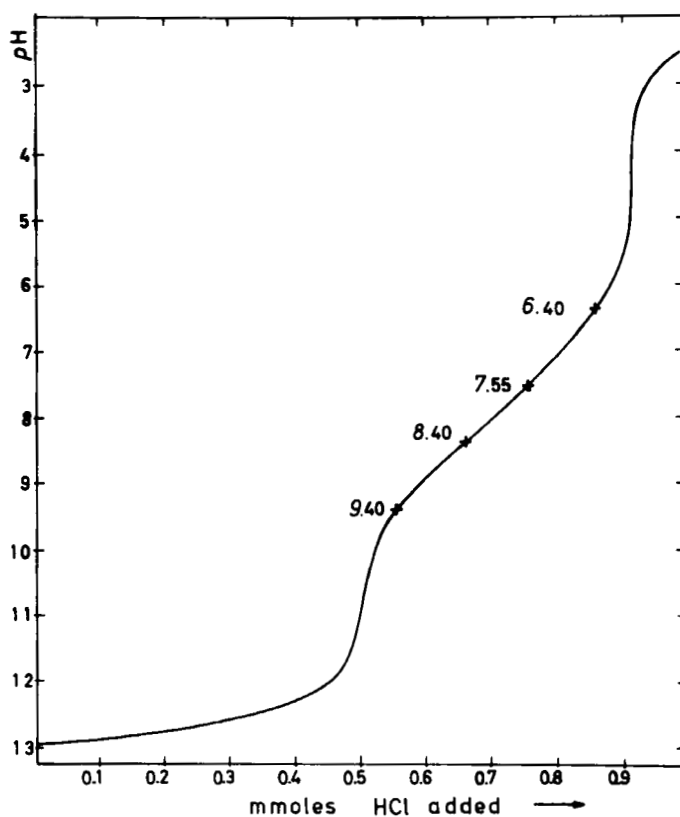


Fig. 2. Electrometric titration curve of kanamycin A free base.

For each of these $[\alpha]_D$ values the molecular rotations (M_D) were calculated. The M_D , calculated from the specific rotation of the free base measured in the authors' laboratory in 0.2N H_2SO_4 , is almost identical to that of the monosulfate. The M_D calculated from the value of Cron et al.²¹ is somewhat lower.

2.3. Electrometric Titration Curve-pK Values

Apparent pK_a values of 6.40, 7.55, 8.40, and 9.40 were derived from the electrometric titration curve of kanamycin A given in figure 2. The curve was determined²³ with an automatic Radiometer titration assembly (TTT 1 and SBR 2) for an aqueous solution (5 ml) of 0.1 mmol kanamycin A free base and 0.5 mmol KOH. Titration was carried out with HCl 0.5N.

2.4. Crystal Properties

The X-ray powder diffraction pattern obtained²⁴ for a commercial sample of kanamycin monosulfate monohydrate containing 2 to 3 % of the B component is presented in Table III.

Experimental conditions

Philips PW 1050/25 vertical goniometer, supplied with flat

rotation specimen holder PW 1064/20

Generator : PW 1130/00 60 kV-3kW

2 kW normal focus Cu tube : 40 kV-40 mA

Divergence slit : 1°

Receiving slit : 0.1°

No beta filter

Focusing monochromator : PW 1966/40

Proportional counter

PHS employed; F.S.D. 4×10^3 cps; time constant 1 s

Scanning speed : 0.5° 2θ per minute

Chart speed : 10 mm/min.

Table III. X-Ray Powder Diffraction Data

d^* (Å)	I/I_o^{**}	d^* (Å)	I/I_o^{**}	d^* (Å)	I/I_o^{**}
12.450	10	3.345	10	2.362	8
7.242	21	3.264	6	2.329	4
7.126	12	3.232	8	2.304	5
6.317	24	3.198	11	2.290	7
6.215	28	3.164	12	2.273	6
5.965	24	3.110	31	2.232	4
5.090	22	3.038	4	2.183	5
5.039	16	2.983	3	2.141	2
4.882	100	2.943	7	2.116	1
4.783	41	2.898	2	2.100	1
4.658	9	2.871	2	2.077	2
4.599	7	2.834	7	2.052	3
4.506	10	2.805	2	2.029	2
4.142	47	2.759	11	1.993	5
4.101	58	2.694	2	1.973	3
4.004	6	2.644	2	1.899	4
3.842	6	2.599	5	1.848	3
3.793	2	2.556	8	1.805	2
3.725	7	2.522	6	1.756	2
3.640	41	2.488	3	1.734	2
3.555	16	2.436	6	1.678	2
3.484	26	2.414	5		
3.466	20	2.374	7		

* $d = \frac{n\lambda}{2 \sin\theta}$ = interplanar distance

** I/I_o = relative intensity (based on highest intensity of 1.00).

The crystal structure of kanamycin monosulfate monohydrate and of the isomorphous kanamycin monoselenate monohydrate has been determined by X-ray analysis²⁵.

2.5. Melting Range

The following melting (decomposition) temperatures have been reported :

for the free base of kanamycin A

250°	Maeda ¹²
255° (decomp.)	Claes <u>et al.</u> ²²

for the monosulfate

268-276° (decomp.)	Maeda ¹²
--------------------	---------------------

2.6. Thermal Analysis

The differential scanning calorimetry (DSC) curve shows²⁶ two endotherms (respectively at 120° and 170°) for kanamycin monosulfate. This is in agreement with the results of loss on drying given in section 8.3. However, no transition was noted²⁷ below 250° in the differential thermal analysis (DTA) curve of the monosulfate. This is in apparent contradiction with DSC measurements and with the results of loss on drying.

2.7. Solubility

The free base, the monosulfate and the sulfate of kanamycin A are soluble in water and almost insoluble in organic solvents such as alcohol, acetone, ether ethyl acetate and benzene. The free base is slightly soluble in formamide¹². The following solubilities have been reported.

<u>Kanamycin</u>	<u>Solvent</u>	<u>Solubility</u>	<u>References</u>
monosulfate	water	350 mg/ml	12
		1 part in 8 parts	6,8,23
	50 % aq.MeOH	5 mg/ml	12
acid sulfate	water	1 part in 1 part	6,8

The solubility of kanamycin sulfate in water at various pH values is given in a paper by Granatek et al.²⁸. Solubilities in various solvents are also given in this paper.

3. Synthesis

3.1. Fermentation - Biosynthesis

Kanamycin is produced commercially by fermentation. The isolation of crystalline kanamycin A monosulfate has been described by Maeda¹². In this procedure the antibiotic is extracted from the culture filtrate by adsorption on a cation exchange resin (Amberlite IRC-50) in the sodium form and eluted from the resin with $2N\ NH_4OH$. The eluate is concentrated, adjusted to pH 9 with H_2SO_4 , decolorized over active carbon and adjusted to pH 8.0-8.2 with NH_4OH . Addition of methanol affords a precipitate of the crude crystalline monosulfate monohydrate of kanamycin A, which is recrystallized from water-methanol or water-methylcellosolve. The biosynthesis of kanamycins has been studied by Kojima *et al.*^{29,30}. A review of the biosynthesis of aminocyclitol antibiotics is given by Rinehart *et al.*³¹.

3.2. Chemical Synthesis

Total synthesis of the kanamycins A, B and C was achieved in 1968 by S. Umezawa and coworkers³²⁻³⁴. An alternative independent synthesis of kanamycin A has been reported in a paper by Nakajima *et al.*³⁵.

4. Stability - Degradation

The stability of kanamycin A free base and sulfate has been investigated by Granatek *et al.*²⁸. Unfortunately the authors did not mention whether the monosulfate or the sulfate with another composition was used in their experiments. The following results, taken from their paper, illustrate that both forms are extremely stable as powders. After storage for 4 months at 56° on average no loss of activity

was observed for the free base. That of the sulfate, stored in identical conditions was 4.3 %. In a pH range of 2.6 to 7.9, aqueous solutions of kanamycin showed an average loss of only 3.5 % after storage for 4 months at 56°. These authors observed that solutions are subject to darkening, due to air oxidation. The color change has no effect on the potency. The crystalline monosulfate can be heated as a powder for 6 hr at 150° without loss of activity⁴.

During determination of the structure of kanamycin A acid degradation has been investigated¹². It was found that the antibiotic is almost unaffected by refluxing with 1N HCl in methanol. In 6N aqueous HCl at 100°, 97 % of the biological activity was destroyed after 45 min and kanamycin was almost completely hydrolysed into its three components : 2-deoxystreptamine, 6-amino-6-deoxy-D-glucose and 3-amino-3-deoxy-D-glucose.

Kanamycin, like the related antibiotics neomycin, paraneomycin and gentamicin, is very stable in alkaline medium. No loss in activity was found when these antibiotics were refluxed for 48 hr in 1.9N aqueous NaOH³⁶.

5. Inactivation by Enzymes

Aminoglycoside-modifying enzymes can be found in a wide variety of resistant bacteria and are known to be coded by plasmids. In most cases the enzymatic modification of the antibiotic results in complete inactivation. The three known modifications induced by these enzymes are : N-acetylation, O-phosphorylation, and O-adenylylation. These mechanisms of inactivation have been reviewed by Benveniste and Davies³⁷. A recent article by Haas and Dowing³⁸ describes the isolation and assay of these enzymes. The kanamycin A-modifying enzymes,

Table IV. Kanamycin A Modifying Enzymes

<u>Enzyme</u>	<u>Cofactor</u>	<u>Modification induced in kanamycin A</u>	<u>Other Substrates</u>
Kanamycin acetyl-transferase (KAT)	Acetyl coenzyme A	Acetylation of the 6'-amino group	Neomycins, kanamycin B, gentamicin C _{1a} , gentamicin C ₂ , tobramycin, butirosins, ribostamycin, sisomicin, BB-K8 (amikacin)
Gentamicin acetyl-transferase III (GAT _{III})	Acetyl coenzyme A	Acetylation of the 3-amino group (of deoxystreptamine)	Kanamycins B & C, gentamicins, sisomicin, ribostamycin, tobramycin, lividomycins
Gentamicin adenyl-transferase (GAdT)	ATP	Adenylation of the 3'-hydroxyl group	Kanamycins B & C, gentamicins, tobramycin
Neomycin phospho-transferase I (NPT _I)	ATP	Phosphorylation of the 3'-hydroxyl group	Kanamycins B & C, neomycins, lividomycins, ribostamycin, gentamicins A & B
Neomycin phospho-transferase II (NPT _{II})	ATP	Phosphorylation of the 3'-hydroxyl group	Kanamycins B & C, neomycins, butirosins, ribostamycins, gentamicins A & B

their substrates, co-factors and the modifications induced in the kanamycin molecule are summarized in Table IV. The data presented in this table are taken from references 37 and 38. The application of aminoglycoside-modifying enzymes in the assay of kanamycin and related antibiotics will be discussed in section 9.

6. Mode of Action

The mode of action of kanamycins is similar to that of other aminoglycoside-aminocyclitol antibiotics and has been reviewed by Weisblum and Davies³⁹ and by Gale *et al.*⁴⁰. These drugs inhibit protein synthesis through an interaction with the 30S ribosomal subunit. They also induce a misreading of the codon. The significance of the latter effect for the lethal action of the antibiotic is not clear.

A structure-activity relationship among the aminoglycoside antibiotics is reported by Benveniste and Davies⁴¹.

7. Pharmacokinetics

Earlier work on absorption, distribution and excretion of kanamycin in humans was reviewed by Kunin⁴² in 1966. A comparative pharmacokinetic study of kanamycin and amikacin (a semisynthetic aminoglycoside antibiotic derived from kanamycin A) in dogs and human has been reported recently by Cabana and Taggart⁴³. The kinetic profiles of both antibiotics are almost identical. The results presented in this paper are similar to those obtained in a previous study⁴⁴. In humans, serum concentrations of about 20 µg/ml were observed at 1 hr after a 500 mg intramuscular dose. The plasma half-life of kanamycin is approximately 2.3 hr. Clearance in man was primarily by glomerular filtration, and urinary excretion of the

unchanged antibiotic accounted for 83 % of the dose. No protein binding of kanamycin by human serum was observed⁴⁵⁻⁴⁷. Kanamycin sulfate is poorly absorbed from the gastrointestinal tract and large amounts of kanamycin are recovered in the stools of patients given the drug by mouth⁴².

The distribution of kanamycin in tissues, after parenteral administration, has been studied by several authors⁴⁸⁻⁵⁰.

8. Methods of Analysis

8.1. Identification

Kanamycin generates a violet color when heated with ninhydrin. This color reaction, which is not specific since it is due to the presence of primary amino function, is given as identification test in the Eur. Ph.⁸, Brit. Pharm. Codex 1968⁵¹ and in the Code of Federal Regulations⁷. The characteristic melting point (about 235° with decomp.) of the crystalline picrate salt of kanamycin is also useful as identification test. The procedure is described in the Brit. Pharm. Codex⁵¹ and in the Eur. Ph.⁸.

Thin layer chromatography (TLC) on silica gel H with a solvent system consisting of 3.85 % aqueous ammonium acetate has been described as identification in the Brit. Ph. 1973⁶ (cf. section 8.62, solvent system V). A ninhydrin reagent (solution in butanol) is used for detection.

The TLC system described by Dubost *et al.*⁵² for the semiquantitative determination of the B-component in commercial samples of kanamycin (section 8.62, solvent system VI) is also a specific method for the identification of kanamycin A²³. The chromatography is carried out on Merck pre-

coated silica gel plates with a 15 % aqueous solution of KH_2PO_4 as a solvent system. Spots are visualized by the color reaction with ninhydrin or by a spray consisting of a 0.2 % alcoholic solution of 1,2-dihydronaphthalene and sulfuric acid 9N in a ratio of 1:1, followed by heating for 5 to 10 min at 150° . Differentiation of kanamycin from related aminoglycoside antibiotics is based on R_f values and the color observed after visualization with the 1,2-dihydroxynaphthalene reagent. Kanamycin gives a brown colored spot, whilst blue spots are obtained for paramomycin and neomycin. Merck precoated silica gel plates may be replaced by plates coated with silica gel H containing 1 % carbomer. In the latter case a 7 % aqueous solution of KH_2PO_4 is used as a solvent system^{8,23} (section 8.62, system VII).

8.2. Determination of Sulfate

The limits for the sulfate content (SO_4) are for kanamycin monosulfate, from 15.7 to 17.3 % (Brit. Ph. 1973⁶) and from 15.0 to 17.0 % (Eur. Ph.⁸), for the acid sulfate from 23.0 to 26.0 % (both Pharmacopoeias). A gravimetric assay method has been described in the Brit. Ph. 1973⁶. A facile method for the determination of the sulfate in kanamycin and in related aminoglycoside antibiotics has been reported by Roets and Vanderhaeghe⁵³. In this method the sulfate ion is titrated with BaCl_2 0.1M, using thorin as indicator, after fixation of the kanamycin free base by ion exchange on a column filled with a suitable strongly acidic resin in the H^+ form (e.g. Dowex 50W-X8, 200-400 mesh).

The most convenient method is described in the Eur. Ph.⁸ and consists in the precipitation of the sulfate with a known amount of BaCl_2 in the presence of ammonia, followed by a

titration of the excess of barium ions with sodium edetate. This procedure, which is given below, has been adapted^{3,4} from a complexometric titration described by Anderegg *et al.*⁵⁴. Kanamycin sulfate (0.250 g) is dissolved in 100 ml water and sufficient concentrated ammonia is added to adjust the pH to 11. After addition of barium chloride 0.1M (10 ml) and of phthaleinpurple (0.5 mg), the solution is titrated with 0.1M sodium edetate, adding 50 ml of ethyl alcohol when the color of the solution begins to change. Titration is continued until the violet-blue color completely disappears.

8.3. Loss on Drying

The water present in kanamycin monosulfate monohydrate can only be removed after heating at high temperature. A loss on drying of 2 to 3.5 % was noticed after heating samples for 6 hr at 150°^{3,4} (the calculated amount of water is 3.0 %). X-Ray powder diffraction patterns of samples heated for 6 hr at 150° revealed a transformation into another crystalline form²⁴. Heating for 4 hr at 150° or 6 hr at 120° is not sufficient for the removal of water present.

According to the Brit. Ph.⁶ and the Eur. Ph.⁸ the loss on drying for kanamycin monosulfate is determined after heating for 3 hr at 60° in vacuo (5 mm Hg or less) over phosphorus pentoxide. This treatment does not alter the X-ray powder diagram²⁴. The limits for this loss on drying is 3 % (Brit. Ph.⁶) and 1.5 % (Eur. Ph.⁸). The values actually observed under these conditions vary from 0.2 to 0.7 %.

For kanamycin acid sulfate the same procedure (3 hr at 60° in vacuo over P₂O₅) is recommended in the Brit. Ph. (Add. 1975)² and in the Eur. Ph.⁷. The limit given in both Pharmacopoeias is 5 %.

The water content of kanamycin sulfate has also been determined by the K. Fischer method. Results obtained in different laboratories are not always in agreement with each other. This may be due to the fact that the kanamycin sulfates are almost insoluble in methanol. Methanol may be replaced as a solvent by pyridine or formamide. In these cases the solvents must be strictly anhydrous.

8.4. Microbiological Assay

The minimum potency required by the Brit. Ph. 1973⁶ is 735 I.U. per mg for kanamycin (mono) sulfate and 670 I.U. per mg for the acid sulfate. The requirements of the Eur. Ph.⁸ will be respectively 750 and 670 I.U. per mg. The minimum potency requirements of the FDA⁷ for kanamycin (mono) sulfate is 750 mcg per mg.

Prescriptions for the microbiological assay using the diffusion procedure can be found in different compendia. The Brit. Ph.⁶ recommends as test organisms Bacillus pumilus NCTC 8241, whereas the Eur. Ph.⁸ suggests the use of Bacillus subtilis ATCC 6633 or NCIB 8054, or Staphylococcus aureus ATCC 6538P or NCTC 6571. The FDA⁷ prescribes Staphylococcus aureus ATCC 6538P. Details of the FDA procedure can also be found in refs. 55 and 56. No detailed description of the turbidimetric assay of kanamycin has been published although it is used in some laboratories. For general information about this method see ref. 57.

8.5. Assay of Kanamycin B

The Code of Federal Regulations⁷ described the determination of the B-component in commercial kanamycin samples. The method, which is similar to the procedure origi-

nally reported by Wakazawa *et al.*⁵⁸, is based on the fact that kanamycin B is more resistant to acidic hydrolysis than kanamycin A. Thus the commercial sample is heated for 1 hr at 100° in HCl 6N and the residual antibacterial activity is assayed using *Bacillus subtilis* ATCC 6633. The limit for the B-component given in the Code of Federal Regulations is 5 %.

A method using column chromatography on Dowex 1-X2 ion-exchange resin in the OH⁻ form (section 8.63), using the reaction with ninhydrin as detection method, is described in the Brit. Ph. 1973⁶. The limit for kanamycin B in commercial samples given in this Pharmacopoeia is 3 %.

A limit test for kanamycin B by thin layer chromatography on Merck precoated silica gel plates has been reported by Dubost *et al.*⁵² (section 8.62). The precoated plates can be replaced²³ by silica gel H layers containing 1 % carbomer (Carbopol 934). In this case the percentage of KH₂PO₄ must be lowered from 15 to 7 %. Ninhydrin is used for detection in both systems. The procedure using the carbomer-containing layers is recommended in the Eur. Ph.⁸ as a limit test for the B-component. The intensity of the secondary spot must be lower than that observed for a reference solution consisting of the kanamycins A and B in a ratio 25:1.

Commercial samples show in these systems a third spot with a higher R_f value than that of either kanamycin A or B. The minor components responsible for this spot were identified²² by degradation and mass spectral studies as paromamine and as 6-O-(3-amino-3-deoxy- α -D-glucopyranosyl)deoxystreptomamine.

8.6. Chromatographic Analysis

8.61. Paper Chromatography

The solvent system of Peterson and Reinecke⁵⁹, which consists of water-saturated butanol containing 2 % p-toluene sulfonic acid, has been used by a number of authors^{1,21,60-63} for differentiation of kanamycin A from the B- and C-components. The R_f values 0.12-0.18 for kanamycin A, 0.26-0.28 for kanamycin B and 0.20-0.24 for kanamycin C have been reported by Rothrock *et al.*⁶¹ (descending chromatography of 40 to 48 hr on Whatman no. 1 paper with the Peterson and Reinecke solvent system). Kanamycins were visualized by bioautography^{1,60}, ninhydrin reaction^{62,63} and "chromato red" staining⁶¹. Another system used for differentiation of the three kanamycins was reported by Kojima *et al.*³⁰. It consists of n-butanol-pyridine-acetic acid-water (6:4:1:3) (v/v) (descending chromatography for 5 days at 20-25°).

Differentiation of kanamycin from related antibiotics by paper chromatography using a combination of several solvent systems has been described in a number of papers⁶⁴⁻⁶⁷. A review article on paper chromatography of antibiotics has been published recently⁶⁸.

8.62. Thin Layer

Various TLC systems for separation of kanamycin A from its congeners (kanamycin A and B) and from other water-soluble basic antibiotics have been reported. Details are given in Table V. Separation of kanamycin A from tuberculo-static antibiotics such as rifamycin SV, capreomycin, viomycin, cycloserine, and streptomycin (or dihydrostreptomycin) has been reported by Voigt and Maa Bared⁶⁹.

Table V. Thin-Layer Chromatography of Kanamycins and Other Water-Soluble Basic Antibiotics (Rf x 100)

System	Plate	Revelation	Reference	Kanamycin A	Kanamycin B	Kanamycin C	Neamine	Neomycin*	Paromamine	Paromomycin*	Gentamicin C*	Spectinomycin	Streptomycin	Dihydrostrept.	Viomycin	Capreomycin	Polymyxin B	Bacitracin
I	SG-1	P	70	65				51		68					11			
I	SG-2	P-N	71	55			52	<u>33</u> , 36	71	49	52, <u>57</u> , 60			0	5	9	66	99
II	SG-1	P	70	55				46		40								
III	SG-3	N	72	38	56	51		<u>26</u> , 29		34	71, 76		0	0				
III	SG-2	N-Cl-P	23	47			50	32		42	66, 62	54	0	0	5	10	81	97
IV	SG-4	N	52	20	3			3					57					
V	SH	P-N	71	48			45	17	58	35	48			53	6	18	0	0
VI	MP	N-Cl-NR-PG	23, 52	43	30	43	38	21	55	33	19, 24	54	60	57	57	37	0	0
VII	SH-C	N-NR-PG-OR	8, 23	36	20		34	9		25	18	58	58					
VIII	SK	DN-NP	73	42				16		38	25		42					
IX	SK	DN-NP	73	33				12		26	18		37					
X	SK	DN-NP	73	34				14		29	15		52					
XI	SK	DN-NP	73	52				36		49	31		29					
XII	KG	MS	74	83				95	91		46		90	91	83		10	0
XIII	C-1	N-ON	75	17	15	23		10		15	20, 28, 35		44	44	21			
XIV	C-2	N	76	48	43		61	<u>52</u> , 24		<u>55</u> , 26			72					
XV	IE	N-CT	77	39			28	15	52	32	5		49	44	25	5	0	1

* If the components of neomycin, paromomycin or gentamicin are separated, the Rf of the major component is underlined.

Visualization of the antibiotics used in the TLC procedures given in Table V

P = spray of 10 % potassium permanganate followed by a spray of a 0.2 % bromophenol blue solution⁷⁰

N = ninhydrin reagent

Cl = spray of a NaOCl solution containing 0.5 % active chlorine followed, after evaporation of the chlorine, by a spray of a 0.5 % KI solution containing 1 % starch

NR = spray of a 0.2 % naphthoresorcinol (1,3-dihydroxynaphthalene) solution in ethanol, followed by a spray of H_2SO_4 9N and heating for 5 to 10 min at 150°

PG = as NR, but with phloroglucinol instead of naphthoresorcinol

OR = as NR, but with orcinol instead of naphthoresorcinol

DN = p-dimethylaminobenzaldehyde-ninhydrin reagent⁶⁹

NP = sodiumnitroprusside-permanganate reagent⁶⁹

ON = oxidized nitroprusside reagent⁷⁹

CT = chlorine-tolidine reagent⁸⁰

MS = Mathis-Schmitt reagent⁸¹

Solvent systems for the TLC procedures given in Table V

I : Upper layer of CHCl_3 -MeOH-17 % ammonium hydroxide (2:1:1)⁷⁰

II : n-Propanol-pyridine-HOAc-water (15:10:3:10)⁷⁰

III : CHCl_3 -MeOH-28 % ammonium hydroxide-water (1:4:2:1)⁷²
tank saturated overnight

IV : 10 % aqueous solution of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ -MeOH-EtOAc (8:7:3)⁵²

V : NH_4OAc (3.85 g) in water (100 ml) (tank saturated overnight)⁷⁸

VI : Aqueous solution of KH_2PO_4 15 % (tank saturated overnight)⁵²

Results given for this system can only be obtained on Merck precoated plates. It was found that the separating power is mainly due to the presence of a polycarboxylic resin which is used as a hinder in these Merck plates. Similar results can be obtained with system VII in which a small amount of a polycarboxylate resin (carbomer) is added to the silica gel^{8,23}.

- VII : Aqueous solution of KH_2PO_4 7 % (tank saturated overnight)^{8,23}
- VIII : n-Propanol-EtOAc-water-25 % ammonium hydroxide-pyridine-3.85 % in water (100:20:60:20:10:200)⁷³
- IX : EtOH-EtOAc-water-25 % ammonium hydroxide-pyridine-3.85 % NH_4OAc in water (100:20:60:10:200)⁷³
- X : MeOH-EtOAc-water-25 % ammonium hydroxide-pyridine-3.85 % NH_4OAc in water (100:20:60:20:10:200)⁷³
- XI : 25 % Ammonium hydroxide-water- Me_2CO (16:144:40)⁷³
- XII : Water-sodium citrate-citric acid (100:20:5)⁷⁴
- XIII : n-Propanol-pyridine-HOAc-water (15:10:3:12)⁷⁵
- XIV : MeCOEt-MeOH-isopropanol-7.9N ammonium hydroxide (10:8:5:3:7) (tank and plate saturated - double development)⁷⁶
- XV : 1.5M NaOAc (adjusted to pH 8.5) containing 1.0M NaCl and 10 % tert-butanol⁷⁷

Plates for the TLC procedures given in Table V

- SG-1 : Silica gel G thickness of layer and mode of activation not specified
- SG-2 : Silica gel G (0.25 mm) activated for 1 hr at 110°
- SG-3 : Silica gel G (0.75 mm) not activated
- SG-4 : Silica gel (0.5 mm) activated for 1 hr at 110°
- SH : Silica gel H (0.25 mm) activated for 1 hr at 110°
- MP : Merck precoated silica gel F-254 plates activated for 1 hr at 110°

- SH-C : Silica gel H containing 1 % carbomer (adjusted to pH 7) activated for 1 hr at 110°
- SK : Silica gel G - kieselguhr G (1:2) activated for 1 hr at 110°
- KG : Kieselguhr G (0.25 mm) activated for 1 hr at 120°
- C-1 : Machery Nagel cellulose powder 300 (0.25 mm) dried for 20 min at 100°
- C-2 : Idem, dried for 2-3 hr at 100-105°
- IE : Dowex 50 x 8 type resin-coated TLC plates in the sodium cycle (Machery Nagel Ionex 25 SA)

8.63. Ion Exchange

Column chromatography of kanamycins and related antibiotics on both acidic and basic ion exchange resins has been reported. Separation on acidic resins is by classical ion exchange chromatography. The separating capacity of strongly basic resins is based on non-ionic adsorption of the antibiotic by the quarternary ammonium groups of the resin. This chromatographic system, which is now referred to as ion exclusion chromatography, was introduced by Rothrock *et al.*⁶¹ for the separation of the kanamycins A, B and C (the order of elution is B, C, A). The procedure permitted isolation of crystalline kanamycin C. Improvements of the original procedure have been reported⁸². Other applications in the field of aminoglycoside antibiotics have been reviewed recently by Umezawa and Kondo⁸³.

Experimental details of ion exclusion chromatography can be found in several papers^{61,72,82-84}. Most of the separations were carried out on Dowex 1-X2 (100-200 mesh) resin (Dow Chemical Co., Midland, Michigan) or on Biorad AG 1-X2 (100-200 mesh) resin (Bio-Rad Laboratories, California) both

in the OH^- form. The resins contain trimethylammonium groups on a polystyrene backbone with a low degree of cross-linking. After application of the antibiotic, the column is developed with CO_2 -free water. Detection systems based on a continuous measurement of electric conductivity, optical rotation and colorimetry after reaction with ninhydrin have been used. High performance liquid chromatography (HPLC) of kanamycin A and B based on ion exclusion has been reported recently on Aminex A-27⁸⁵ and Biorex 9 resins⁸⁶ (Bio-Rad Laboratories, California).

Weakly acidic carboxylate resins, such as Amberlite IRC-50 (Rohm and Haas Co., Philadelphia), are widely used in industry for the isolation of kanamycin and other aminoglycoside antibiotics from culture filtrates¹². The antibiotic is adsorbed on the carboxylic resin in the Na^+ or NH_4^+ and eluted with 1N aqueous ammonium hydroxide. Separation of the three kanamycins and of other minor components present in commercial samples was achieved on the chromatographic grade resin by elution with 0.2N NH_4OH ²². Gradient elution has been used for separation of other aminoglycoside antibiotics⁸³. References for applications of carboxylic-, sulfonic- and phosphonic acid resins, and of cellulose- and sephadex-ion exchangers in extraction and purification of aminoglycoside antibiotics can be found in a review article by Umezawa and Kondo⁸³. High performance liquid chromatographic (HPLC) determination of kanamycins A and B on a pellicular cation exchanger such as Zipax SCX (Dupont) has been reported recently⁸⁷.

8.64. Gas Liquid

Tsuji and Robertson⁸⁸ reported gas chromatographic separation of the O,N-trimethylsilyl derivatives of kanamycins A, B and C on a 3 x 1830 mm glass column packed with 3 % OV-1 on Gas Chrom Q at a temperature of 300° using a flame ionization detector. The volatile derivatives were prepared by silylation (45 min at 75°) of a freeze-dried sample of kanamycin sulfate with N-trimethylsilylimidazole in pyridine (Tri-Sil Z, Pierce, Rockford, Illinois) and N-trimethylsilyldiethylamine. Addition of trilaurin as an internal standard permits quantitative analysis.

Similar conditions were described for TMS derivatives of neomycin and paromomycin and other aminoglycoside antibiotics⁸⁹. The order of elution given in the original paper⁸⁸ is kanamycin B, kanamycin A and kanamycin C. Japanese authors⁹⁰ found that kanamycin C was eluted before kanamycins A and B, under GLC conditions similar to those employed by Tsuji and Robertson. Numerous factors may easily interfere with the GLC determination of neomycin and of other aminoglycoside antibiotics. These have been discussed by Margosis and Tsuji⁹¹. The solution to some of the common problems encountered during GLC analysis of neomycin is given by these authors, and also by Tsuji and Robertson⁸⁹ in a review article on GLC of antibiotics.

GLC of N-trifluoroacetyl-O-trimethylsilyl derivatives of a number of aminoglycoside antibiotics (including the three kanamycins) has been reported by Omoto et al.⁹⁰.

8.7. Electrophoresis

High-voltage paper electrophoresis of kanamycins and other water-soluble basic and amphoteric antibiotics has

been described by Maeda *et al.*⁹². The spots of the kanamycins were visualized with ninhydrin. Mobilities relative to alanine (R_m values) are 1.82 for kanamycin A, 1.92 for kanamycin B and 1.85 for kanamycin C. In a review article⁹³ on electrophoresis of antibiotics by two of the authors of the original paper somewhat different R_m values are given (1.74, 1.89 and 1.70, respectively for the kanamycins A, B and C). Electrophoretic separation of aminoglycoside antibiotics including kanamycin A has been reported by Ochab⁹⁴. Resolution of antibiotic mixtures in serum samples by high-voltage electrophoresis on agarose is described by Reeves and Holt⁹⁵.

9. Determination in Body Fluids

Since kanamycin acid, like other aminoglycoside antibiotics, may cause ototoxicity and renal impairment, it is advisable to monitor the antibiotic level in the serum of patients receiving these drugs. Special and rapid assay procedures have been worked out for this purpose. Sabath *et al.*^{96,97} described a microbiological assay method. Interference by penicillins and cephalosporins can be eliminated by a treatment of the serum with a "broad-spectrum" β -lactamase^{96,98}. A method based on the inhibition by aminoglycoside antibiotics of the urease activity of *Proteus* sp. has been reported by Noon *et al.*⁹⁹. A semiquantitative determination of kanamycin in serum and urine, based on a visual comparison of fluorescent intensity with that of reference samples on TLC plates after reaction with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, has been developed by Benjamin *et al.*¹⁰⁰. Enzymatic assays employing aminoglycoside-modifying enzymes (section 5) have been introduced recently^{101,102}. In these procedures the antibiotic is enzymatically modified in the

presence of a radiolabeled cofactor. Kanamycin acetyltransferase (KAT)¹⁰¹ and gentamicin acetyltransferase¹⁰² have been used in the assay of kanamycin.

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KETAMINE

William C. Sass and Salvatore A. Fusari

Contents

1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor
2. Physical Properties
 - 2.1 Spectral
 - 2.11 Infrared Spectrum
 - 2.12 Nuclear Magnetic Resonance Spectrum
 - 2.13 Ultraviolet Absorption Spectrum
 - 2.2 Mass Spectrum
 - 2.3 Differential Thermal Analysis and Melting Point
 - 2.4 Solubility
 - 2.5 Optical Rotation
 - 2.6 Ionization Constant
 - 2.7 Crystal Properties
 - 2.71 Derivative Crystallinity
 - 2.72 X-Ray Diffraction
3. Synthesis
4. Decomposition
 - 4.1 Metabolic Decomposition
 - 4.2 Chemical Decomposition
5. Methods of Analysis
 - 5.1 Elemental Analysis of the Hydrochloride
 - 5.2 Ion-Pairing Colorimetric and Fluorescence
 - 5.3 Ultraviolet
 - 5.4 Differential Thermal Analysis
 - 5.5 Non Aqueous Titration
 - 5.6 Tritium Labeling
 - 5.7 Chromatography
 - 5.71 Paper Chromatography
 - 5.72 Thin Layer Chromatography
 - 5.73 Gas Chromatography

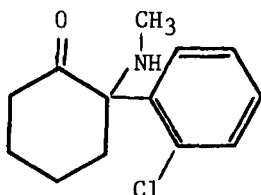
5.74 Liquid Chromatography

6. Determination in Body Fluids
7. References

1. Description

1.1 Name, Formula, Molecular Weight¹

Ketamine is 2-(2-chlorophenyl)-2-(methylamino)cyclohexanone. The hydrochloride bears the clinical investigation number CI-581.



Molecular formula: $C_{13}H_{16}ClNO$

Molecular weight: 237.74

Molecular formula of the hydrochloride:
 $C_{13}H_{16}ClNO \cdot HCl$

1.2 Appearance, Color, Odor

Ketamine and the hydrochloride are both odorless, white crystalline powders.²

2. Physical Properties

2.1 Spectral

2.11 Infrared Spectrum

Infrared spectra of the base in chloroform (Figure 1) and of the hydrochloride as a 0.5% dispersion in potassium bromide (Figure 2) were obtained⁶ with a Perkin-Elmer Model 621 grating infrared spectrophotometer. The high energy absorption between 2600 and 3000 cm^{-1} of the hydrochloride has been ascribed⁵ to the amine hydrochloride while that at 1730 and 780 cm^{-1} result from carbon-oxygen stretching and phenyl-hydrogen bending respectively. (1200 cm^{-1} is $CHCl_3$)

2.12 Nuclear Magnetic Resonance Spectrum

Figure 3 shows the proton magnetic

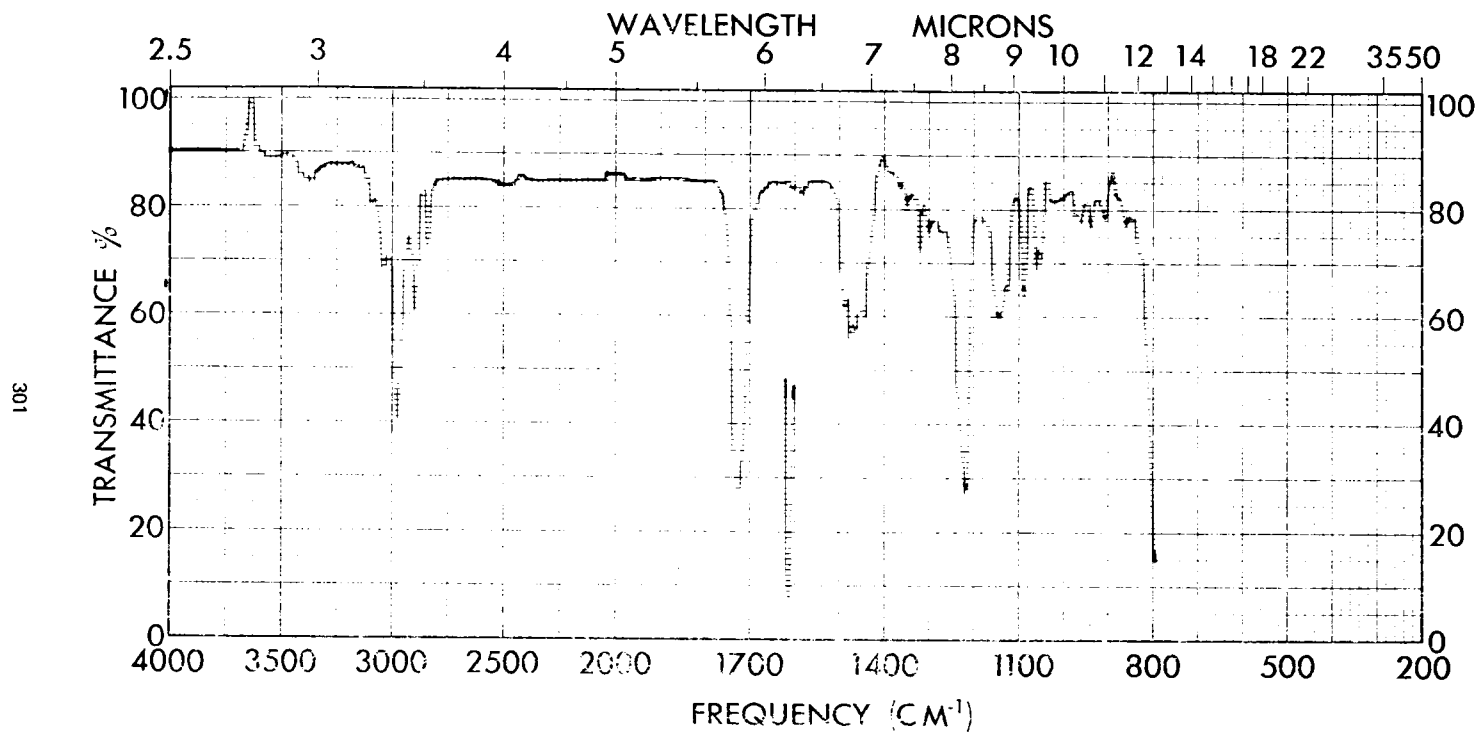


Figure 1. Infrared Spectrum of Ketamine Base in Chloroform.
(1601.0 cm⁻¹ is polystyrene reference peak)

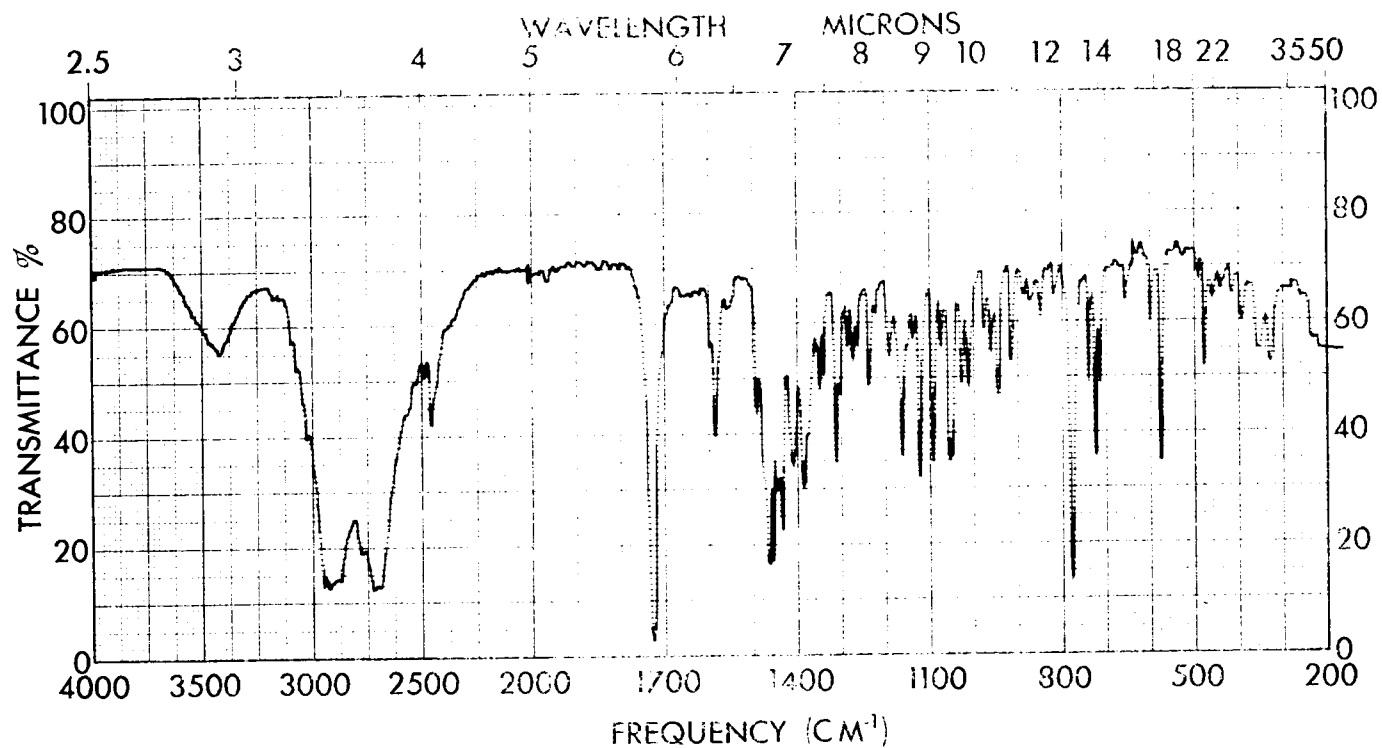


Figure 2. Infrared Spectrum of Ketamine Hydrochloride in KBr.

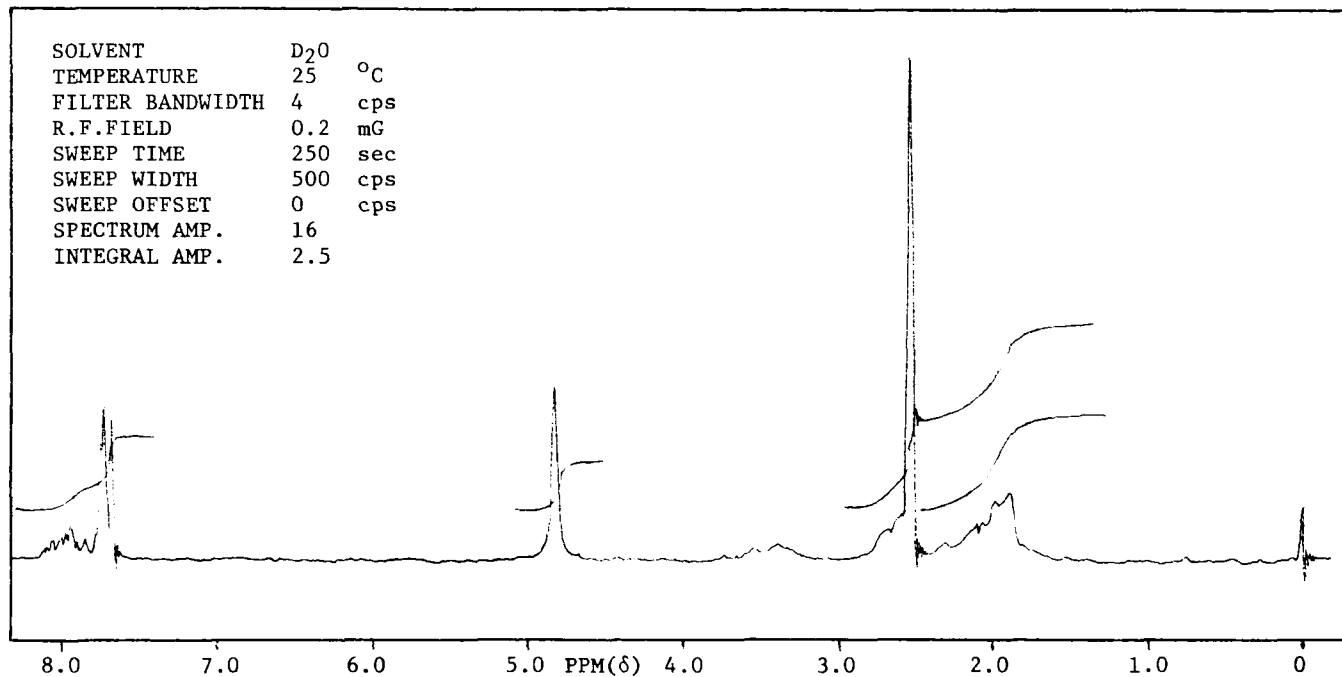


Figure 3. 60 MC NMR Spectrum of Ketamine Hydrochloride in D₂O.

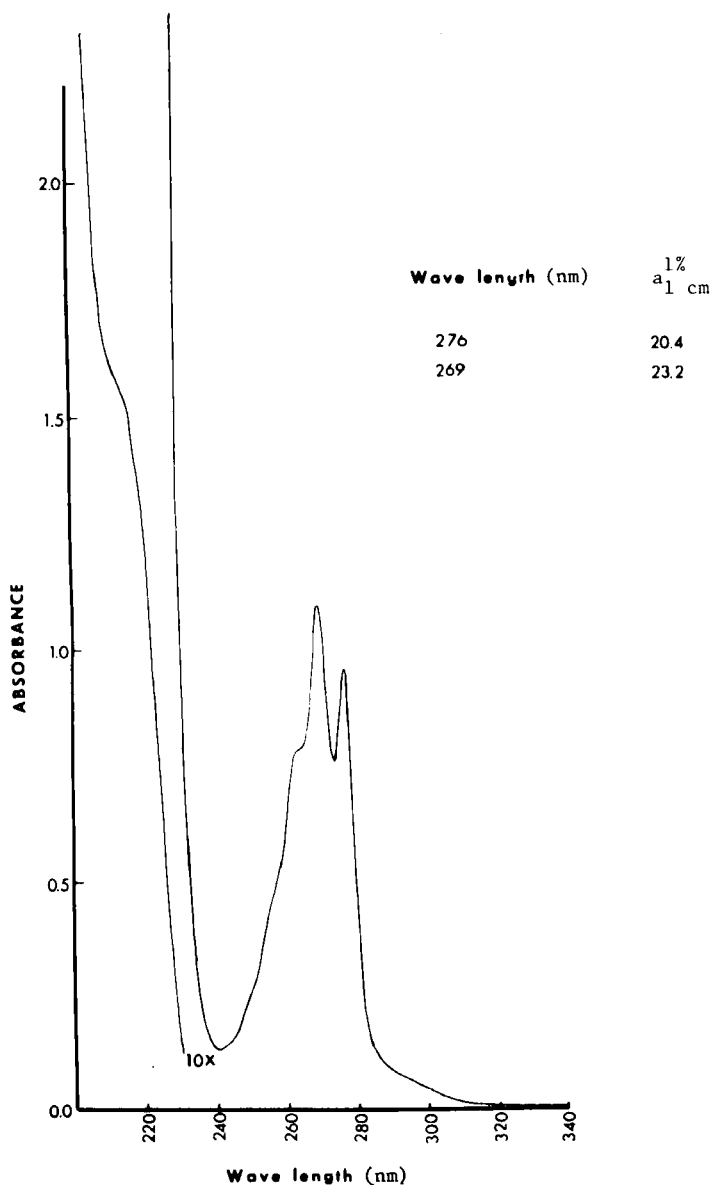


Figure 4. Ultraviolet Spectrum of Ketamine Hydrochloride in 0.1 N Hydrochloric Acid.

resonance spectrum of ketamine hydrochloride in D₂O at 60 meg. Hz. The following assignments have been made:⁴

Structural Assignments

<u>σ ppm</u>	<u># of Protons and Description</u>
2.0	5 - protons of cyclohexanone ring. Shape of absorption peak is typical of cyclohexyl ring protons
2.6	5 - Sharp peak is N-CH ₃ , rounded peak at 2.7 ppm represents 2 protons of cyclohexanone ring
3.5	1 - One of protons on cyclohexanone ring. This proton is most probably on the carbon α to the carbon bearing -N-CH ₃ Hydrogen bonding of this proton to N would lower its chemical shift
4.8	2 - Two protons. Total integration is 19 spaces; subtract 5 spaces for D ₂ O blank to give 14 spaces or two protons. These are exchangeable protons so that they are -NH and H-Cl protons
7.7	4 - Aromatic ring protons

2.13 Ultraviolet Absorption Spectrum

Figure 4 is the ultraviolet spectrum⁵ of ketamine hydrochloride in 0.1N hydrochloric acid obtained on a Cary 15. The two maxima at 276 and 269 nm. represent a(1%, 1 cm.) values of 20.4 and 23.2 respectively.

In 0.1N sulfuric acid, maxima at 264 nm. (a 1%, 1 cm. = 16.6), 269 nm. (a 1%,

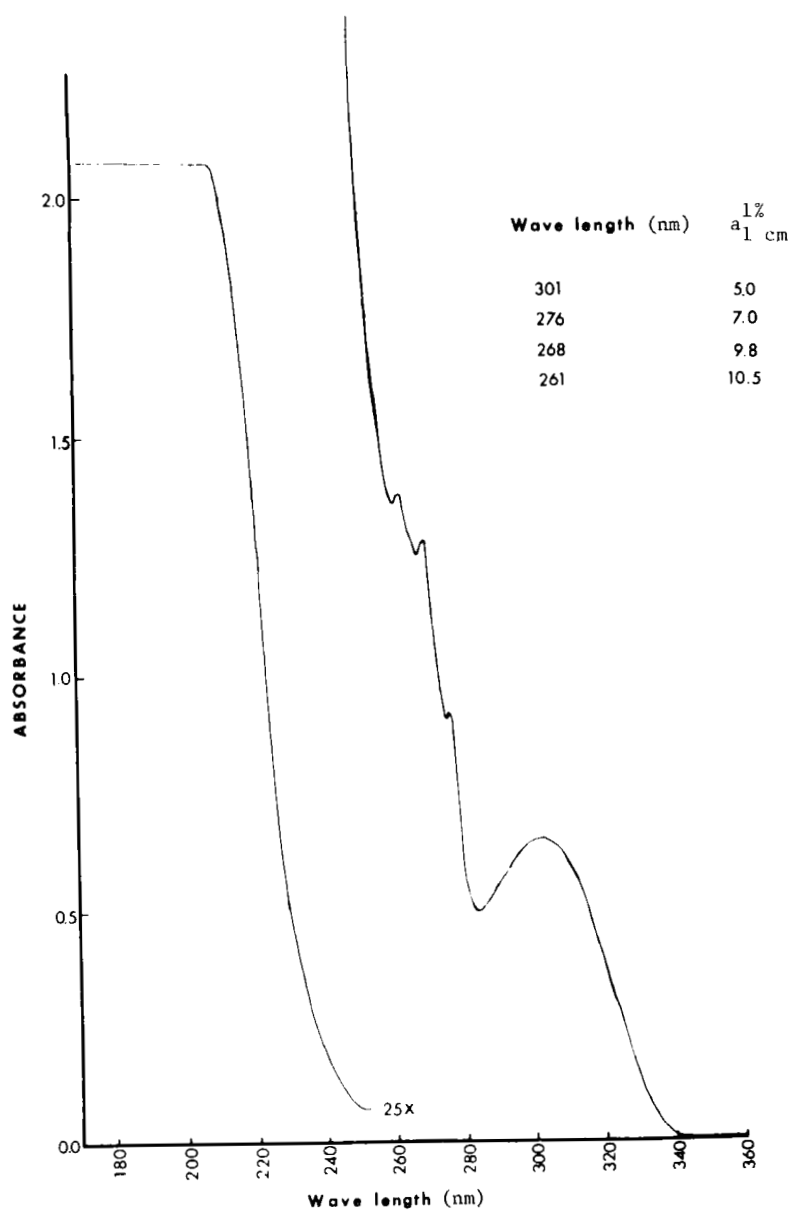


Figure 5. Ultraviolet Spectrum of Ketamine Base in 95% methanol - 0.01N sodium hydroxide.

1 cm. = 23.2), and 276 nm. (a 1%, 1 cm. = 20.3) have been reported.²⁴

Figure 5 represents the alkaline spectrum (0.01N sodium hydroxide in 95% methanol) with the following a(1%, 1 cm.) values: 301 nm. (5.03); 276 nm. (7.07); 268 nm. (9.80); and 261 nm. (10.58).

2.2 Mass Spectrum

Although the mass spectrum of the hydrochloride cannot be easily obtained because of its low volatility, the normalized fragmentation pattern of the base^{6,30} is shown in Figure 6. Tabulated values are ± 0.5 mass unit. The pattern is consistent with a progressive loss of C_2H_4 (209), CO (181), and CH_2NH (152). Fragments at 211, 183, and 154 would result from the chlorine isotope.

2.3 Differential Thermal Analysis and Melting Point

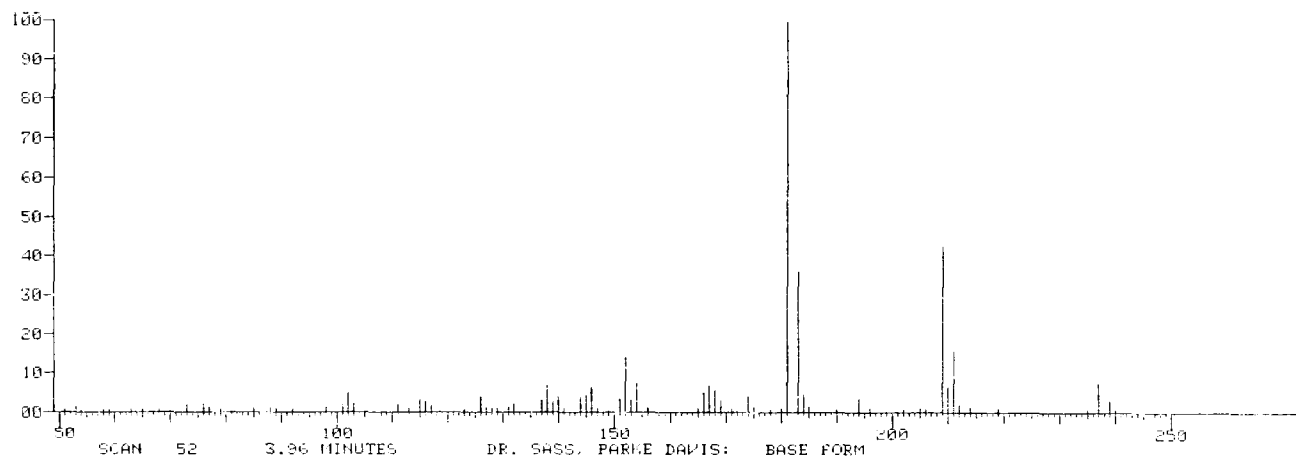
Ketamine reportedly melts at 92-93⁰¹. A differential thermal analysis thermogram⁶ of the base run on a Mettler DTA (Figure 7) displays only a single melting endotherm at 92.25°C. The heat of fusion was found to be -25.81 m cal/mg. The observed specific heat at 90⁰ is 1.9 m cal/mg. °C. Decomposition of the hydrochloride precludes a precise determination of thermodynamic properties.

2.4 Solubility⁵

One gram of the hydrochloride will dissolve in:

- 6 ml. of methanol
- 14 ml. of 95% ethanol (USP)
- 60 ml. of chloroform
- 60 ml. of absolute ethanol

One gram of the hydrochloride is incompletely dissolved in 60 ml. of acetone, ether, benzene, DMF, or dioxane.



RAW DATA
 DR. SASS, PARKE DAVIS: BASE FORM 16-JUN-76
 A 52 TIC = 0118 3.96 MIN
 RANGE 50 THRU 250 THRESHOLD = 0.00

M/E	R I	M/E	R I	M/E	R I	M/E	R I	M/E	R I	M/E	R I	M/E	R I	M/E	R I
50.650	0.16	76.302	0.34	103.201	0.52	130.949	0.36	146.075	0.26	169.185	0.00	184.082	0.36	210.357	1.68
53.250	0.36	77.117	0.30	110.984	0.50	131.540	0.30	148.933	0.18	171.283	0.16	190.154	0.16	211.408	4.04
54.259	0.20	78.632	0.18	112.902	0.28	132.378	0.52	150.839	0.82	174.244	1.04	193.675	0.92	212.269	0.48
57.976	0.16	80.712	0.16	114.900	0.66	137.033	0.74	151.681	3.62	175.468	0.36	194.140	0.26	213.535	0.36
59.177	0.16	85.060	0.26	116.199	0.64	137.982	1.74	152.710	0.80	178.322	0.16	196.125	0.26	215.148	0.24
62.898	0.14	87.689	0.24	117.302	0.62	138.982	0.70	154.312	1.94	179.666	0.50	201.613	0.14	235.324	0.16
64.818	0.18	89.375	0.20	123.072	0.16	140.103	1.02	156.058	0.24	180.619	26.10	205.082	0.26	237.473	1.02
67.814	0.14	91.548	0.18	125.630	1.06	141.351	0.24	164.837	0.24	181.433	3.86	206.263	0.18	238.640	0.50
70.302	0.18	98.173	0.34	127.484	0.36	143.902	0.92	165.814	1.30	183.101	9.40	208.590	1.26	239.482	0.78
72.537	0.50	101.466	0.42	128.392	0.28	144.088	1.12	166.820	1.72	184.089	1.52	209.432	11.14	240.422	0.16
75.595	0.52	102.232	1.28	129.445	0.20	146.355	1.66	168.130	1.50						

Figure 6. Mass Spectrum of Ketamine Base

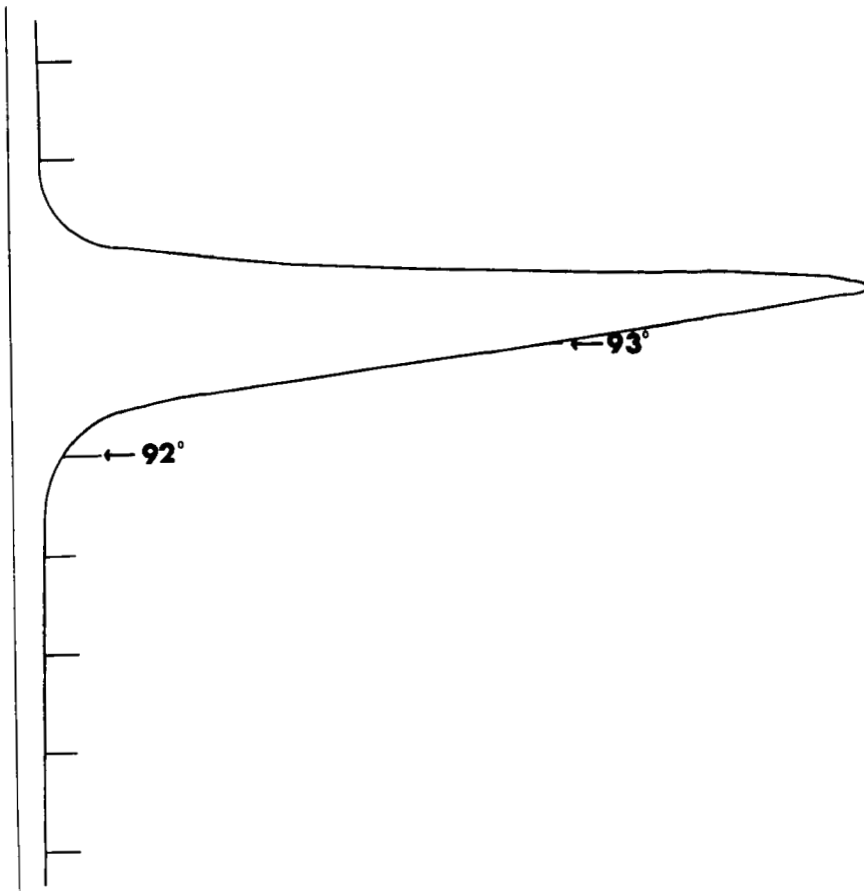


Figure 7. Differential Thermal Analysis Melting Curve.

2.5 Optical Rotation

Isolation of the d(+) isomer of the hydrochloride from a racemic mixture using d-camphorsulfonic acid⁷ resulted in a compound with a specific rotation of $(\alpha)_D^{20} = +90^{\circ}$ (0.98% in methanol). Other physical⁸ and physiological⁹ properties were similar to unresolved commercially available material.

2.6 Ionization Constant

The pK_a of ketamine and the N-dealkylated metabolite are³¹ 7.5 and 6.65. The pH of 10, 50, and 100 mg./ml. solutions⁹ of the hydrochloride are 4.63, 4.16, and 3.92 respectively.

2.7 Crystal Properties

2.71 Derivative Crystallinity²⁴

In platinic iodide solution, rhomboidal plates are formed (sensitivity to 1 in 1000 solution). With potassium bismuth iodide solution, small plates are formed (also sensitivity to 1 in 1000).

2.72 X-Ray Diffraction

X-Ray Diffraction values on the hydrochloride obtained on a Norelco Diffractometer^{6,29} using Copper K₂ radiation ($\lambda = 1.5418$) and a crystal monochrometer are listed in Table I. Variations in the X-Ray pattern of the base suggest that polymorphism may occur.

TABLE I
X-Ray Diffraction of Ketamine Hydrochloride

$\underline{d\text{\AA}}$	100(I/I ₁)	$\underline{d\text{\AA}}$	100(I/I ₁)
9.70	5.8	5.30	1.4
7.43	100.0	4.87	42.1
6.92	3.0	4.63	5.9
6.44	15.6	4.55	12.1
6.14	11.6	4.32	1.5
5.90	1.8	4.14	7.9

λ	$100(I/I_1)$	λ	$100(I/I_1)$
4.13	20.4	2.82	3.6
3.72	81.7	2.72	4.4
3.57	2.4	2.70	35.2
3.45	5.9	2.63	2.7
3.35	3.1	2.44	3.4
3.25	7.6	2.11	1.8
3.22	3.7	2.02	4.0
3.17	5.7	1.83	1.8
3.15	3.6	1.79	2.6
3.05	1.6	1.75	2.6
2.92	7.0	1.64	2.6
2.90	13.2	1.44	1.5

3. Synthesis

3.1 Ketamine hydrochloride may be prepared¹⁰ from o-chlorobenzaldehyde by the procedure³ shown in Figure 8.

4. Decomposition

4.1 Metabolic Decomposition

An initial rapid drop in human plasma levels (half-life 10 min.¹¹, 11 min.²⁶, 17 min.¹², and 25 min.¹³) due to distribution of drug to the tissues is followed by a first order decrease in plasma levels with a half-life of about 2.5 hours.^{11,26}

Describing the absorption pharmacokinetic behavior of ketamine following intravenous injection by a two-compartment model, the half-life of the β -phase has been reported²⁵ as 2.52 hr., 3.99 ± 1.23 hr., and 6.84 ± 2.97 hr. for ketamine, N-dealkylated amine, and the dehydro-N-dealkylated metabolites respectively.

In addition to small amounts of the intact drug excreted, the decomposition scheme shown in Figure 9 has been suggested.^{11,15,25} No indication of protein binding was observed.¹¹ Another report²⁵ suggests that if present, protein binding does not exceed 12%.

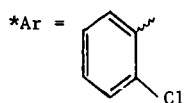
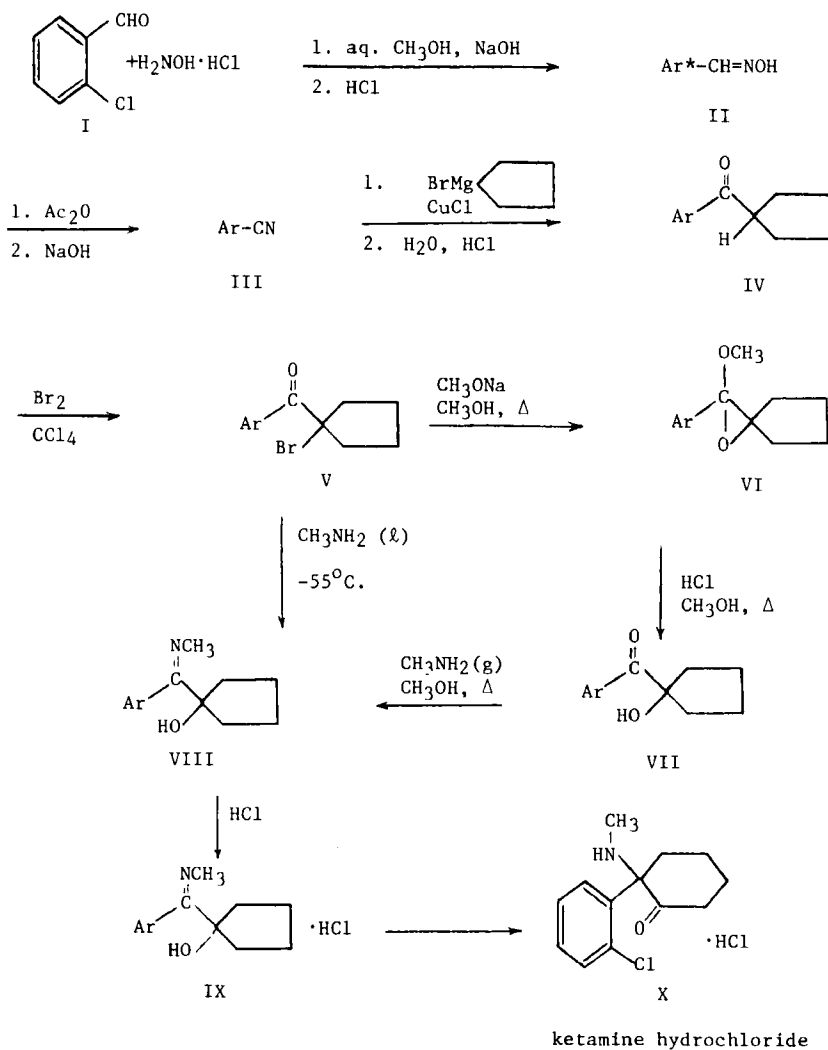


Figure 8. Synthetic Procedure

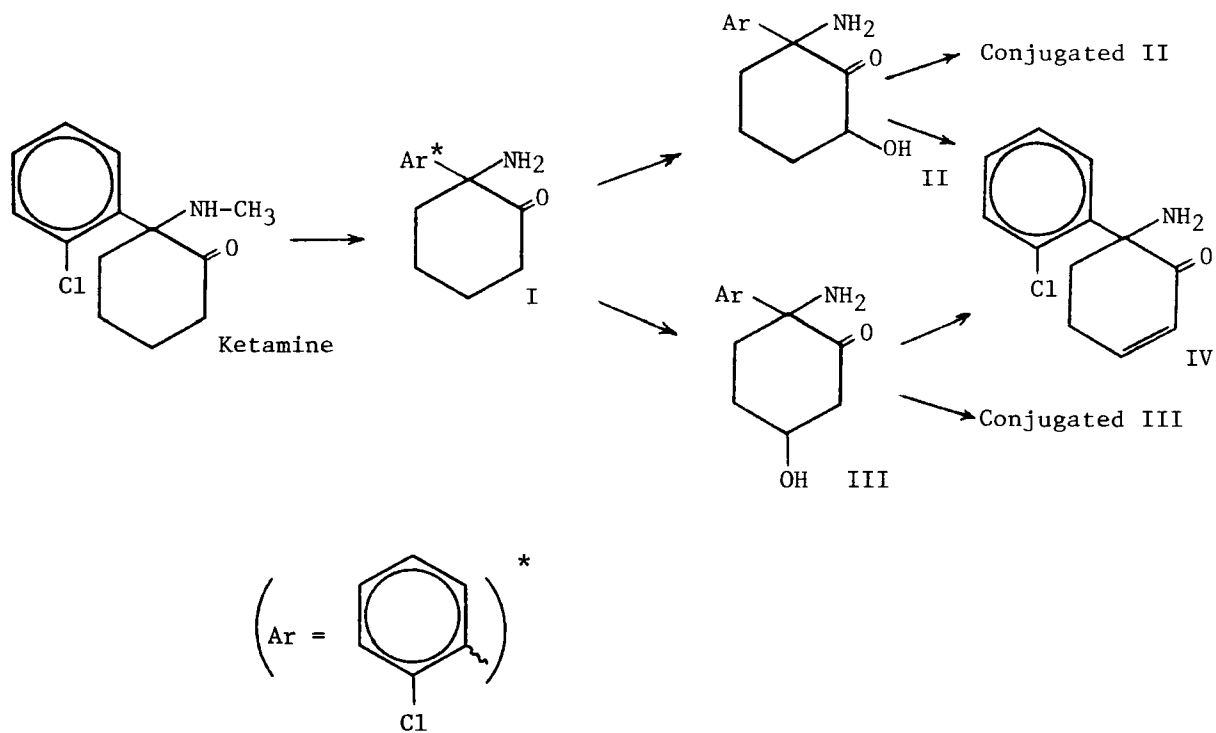


Figure 9. Metabolic Decomposition.

4.2 Chemical Decomposition

Ketamine in aqueous solution has been shown¹⁴ to react under accelerated conditions of high temperature and pH by a process which involves initial formation of 1-[(2-chlorophenyl)(methyylimino)methyl]cyclopentanol (I)(Figure 10). This intermediate, depending on temperature and pH, may then isomerize back to Ketamine or hydrolyze to (2-chlorophenyl)(1-hydroxycyclopentyl)methanone (III), the primary product of this reaction. 2-(2-chlorophenyl)-2-hydroxycyclohexanone (IV) which may be a major, although not primary, product results from isomerization of the cyclopentyl hydroxyketone (III).

When the accelerating conditions are avoided, aqueous solutions and the powder exhibit extraordinary stability.¹⁴

5. Methods of Analysis

5.1 Elemental Analysis of the Hydrochloride

<u>Element</u>	<u>Found</u> ³	<u>Theory</u>
% C	57.05-57.29	56.94
% H	6.49-6.61	6.25
% N	4.95	5.11
% Cl (total)	25.88-26.02	25.86
% Cl (ionic)	13.06	12.93

5.2 Ion-Pairing Colorimetric and Fluorescence

Ion pair extraction into an organic phase using methyl orange¹⁵ is reported to be a less sensitive method than extraction with xylene red B into 1,2-dichloroethane followed by fluorescence analysis.¹⁶ Excitation and emission wavelengths of 562 and 578 nm. were used.¹⁶ With a modification of the xylene red B procedure¹⁷, atropine, diazepam, pentobarbital, fluothane, oxytocin, and ergometrin have been shown not to interfere with the assay, although two of the ketamine metabolites do.

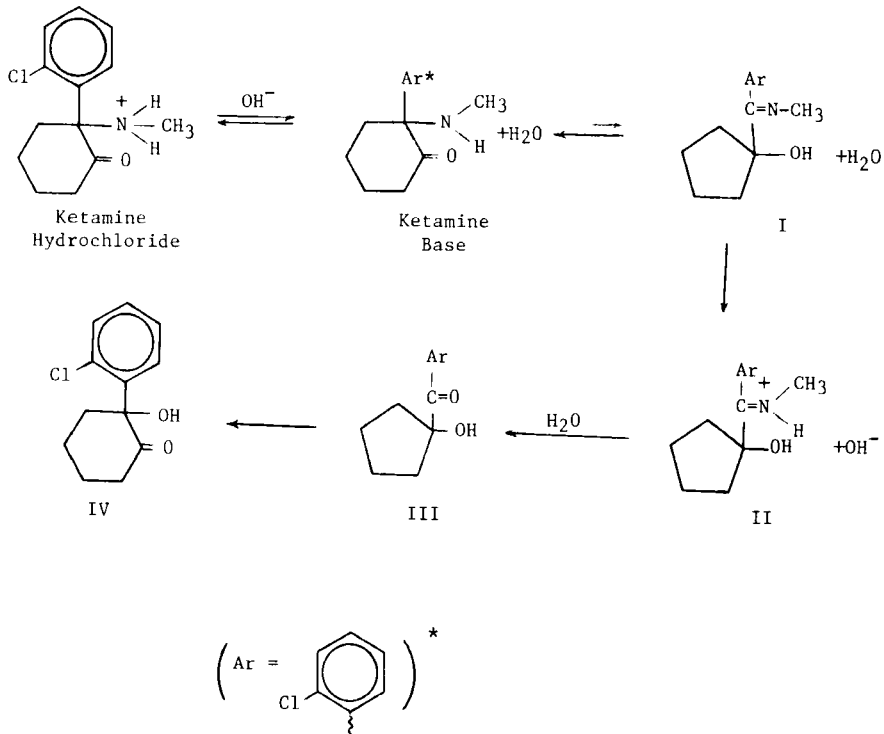


Figure 10. Chemical Decomposition

5.3 Ultraviolet

In the absence of interfering substances, ketamine may be analyzed directly by ultraviolet spectroscopy.⁵

5.4 Differential Thermal Analysis

Pure base may be analyzed by thermal analysis.⁶ Figure 7 is a thermogram of a recrystallized sample which contains less than 1×10^{-3} mole % impurity.

5.5 Non Aqueous Titration

A sample dissolved in glacial acetic acid containing mercury (II) acetate may be titrated with 0.1N perchloric acid in glacial acetic acid to the blue-green end point of crystal violet.⁵

5.6 Tritium Labeling

Heating of ketamine hydrochloride to 100°C. with trifluoroacetic acid and tritiated water in the presence of pre-reduced platinum catalyst for 18 hours formed the labeled product with at least 77% tritium incorporation alpha to the carbonyl.¹⁸ Labile tritium should be removed by treatment with strong alkali¹⁸ to avoid tritium incorporation in body water. Labeled ketamine hydrochloride has been used to study metabolic decomposition.^{11,15}

5.7 Chromatography

5.71 Paper Chromatography²⁴

A 2.5 μ l. spot of a 1% solution in 2N acetic acid is applied to Whatman No. 1 paper previously dipped in a 5% sodium dihydrogen citrate solution, blotted, and dried. Development in an unequilibrated chamber with a solution of 4.8 grams of citric acid in 130 ml. of water plus 870 ml. of n-butanol resulted in a zone at Rf 0.55 which was visible under ultraviolet light after spraying with iodoplatinate or bromocresol green solution.

5.72 Thin Layer Chromatography

Two of the four tritium labeled metabolites and intact ketamine hydrochloride have been separated on Silica Gel GF using chloroform: ethyl acetate:methanol:ammonium hydroxide (60:35:5:1). The intact molecule at $R_f = .65$ and metabolites were detected by their radioactivity.¹¹ Separation of the unresolved metabolites¹⁹ was accomplished on Aluminum Oxide HF using chloroform: cyclohexane:diethylamine (60:40:2). Chloroform: cyclohexane:ethyl acetate:ammonia (25:50:25:5) has been used^{25,27} to separate ketamine ($R_f = 0.58$) and the N-dealkylated metabolite ($R_f = 0.41$) on a LQ6D plate. The other major metabolite is separated but exists as a diffuse zone. All were visualized by exposure to iodine.

A system⁵ used to separate ketamine hydrochloride and (2-chlorophenyl)(1-hydroxycyclopentyl)methanone is Kieselgel DF-5 using benzene: methanol:ammonium hydroxide (90:10:1). R_f values of 0.7 and 0.6 respectively are observed for the compounds under 254 and 366 nm. ultraviolet light.

Concentrated ammonium hydroxide in methanol (1.5:100) has also been used²⁴ to develop samples on activated silica gel G. The main zone at R_f 0.72 was made visible with acidified iodoplatinate spray.

5.73 Gas Chromatography

Since gas chromatography allows rapid, quantitative analysis of ketamine and its degradation products, numerous systems have been utilized.^{20,21} The use of all glass systems²² and the avoidance of evaporation to dryness¹³ have been suggested to avoid degradation. Chromatographic conditions employed are summarized in Table II.

5.74 Liquid Chromatography

Reverse phase chromatography on C₁₈ Microbondapak columns using water:acetonitrile (1:1) has been employed²⁸ to separate the p-nitro-

TABLE II

Conditions Used In Gas Chromatographic Separations of Ketamine

<u>Ref.</u>	<u>Column</u>	<u>Column Temp.</u>	<u>Detector</u>	<u>Internal Standard</u>
11	1% ECNSS-M	155°	FID, EC	o-trifluoromethyl and o-Bromo analogs*
12	3% OV-17 3% (100/120 Gas Chrom Q)	195°	E.C. of hepta- fluorobutyryl derivative	o-Bromo analog*
13	1% OV-101 and 3% succinamine polymer on (100/120 Gas Chrom Q)	158°	FID	CL-392
15	1% ECNSS-M (80/100 Gas Chrom P)	170°		o-trifluoromethyl analog*
20	2.5% SE-30 (80/100 Chromasorb G)	200°	FID	Pentobarbital
21	1% DDTS Gas Chrom Q	180°	FID	

TABLE II (Continued)

<u>Ref.</u>	<u>Column</u>	<u>Column Temp.</u>	<u>Detector</u>	<u>Internal Standard</u>
22	0.5% polyethylene-glycol (20,000 M) (80/100 Chromasorb G) silinized	98—180° @ 3°/min.	FID	methyldiphenylamine
23	2½% SE-30 (80/100 Chromasorb G)	200°	FID	Pentobarbital
26	0.5% PEG 20000 M (80/100 Chromasorb G-DMCS)	90—200°C.	FID	Carbothesin
28	10% UCW-982 (80/100 CWAU-DMCS)	270°C.	FID	— (all separated as p-nitrobenzamides)
25, 27	1% Carbowax 20-M (60/80 Gas Chrom G AW-DMCS)	210°C.	FID	—

*analogs of ketamine

benzamide derivatives of ketamine and its metabolites. Derivatization is required to enhance the otherwise low absorbance at 254 nm.

6. Determination in Body Fluids

Ion-pairing^{15,16,17}, tritium labeling^{18,11}, thin layer chromatography^{5,11,19,25,27}, gas chromatography^{11-13,15,20,22,25-28,31} and liquid chromatography²⁸ techniques have been applied to the determination of ketamine and its metabolites from body fluids.

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MINOCYCLINE

Vladimir Zbinovsky and George P. Chrekian

CONTENTS

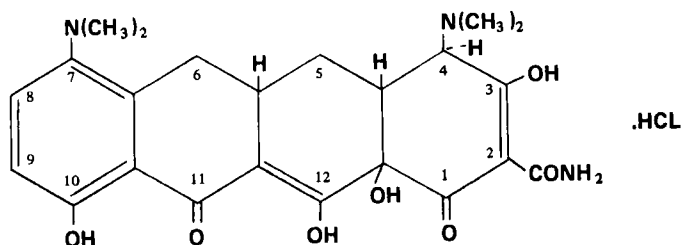
1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor
2. Physical Properties
 - 2.1 Infrared Analysis
 - 2.2 Nuclear Magnetic Resonance Spectrum
 - 2.3 Ultraviolet Spectra
 - 2.4 Mass Spectra
 - 2.5 Optical Rotation
 - 2.6 Thermogravimetric Analysis
 - 2.7 Differential Thermal Analysis
 - 2.8 Solubility
 - 2.9 Solvent Partitioning Data
 - 2.10 Crystal Properties
3. Synthesis
4. Stability, Isomerization, Degradation
5. Pharmacodynamic Studies
6. Methods of Analysis
 - 6.1 Elemental Analysis
 - 6.2 Chromatographic Analysis
 - 6.21 Thin Layer
 - 6.22 Column
 - 6.3 Direct Spectrophotometric Analysis

MINOCYCLINE HYDROCHLORIDE

1. Description

1.1 Name, Formula, Molecular Weight

Minocycline hydrochloride is known chemically as 4,7-bis (dimethylamino)1,4-4a,5,5a,6,11,12a-octahydro-3,10,12,-12a-tetrahydroxy-1,11-dioxo-2-naphthacenecarboxamide mono-hydrochloride and by the trivial name 7-dimethylamino-6-demethyl-6-deoxytetracycline hydrochloride.

 $C_{23}H_{27}N_3O_7 \cdot HCl$

MOL. Wt.: 493.94

1.2 Appearance, Color, Odor

Minocycline hydrochloride occurs as a yellow crystalline powder. It is essentially odorless and has a somewhat bitter taste.

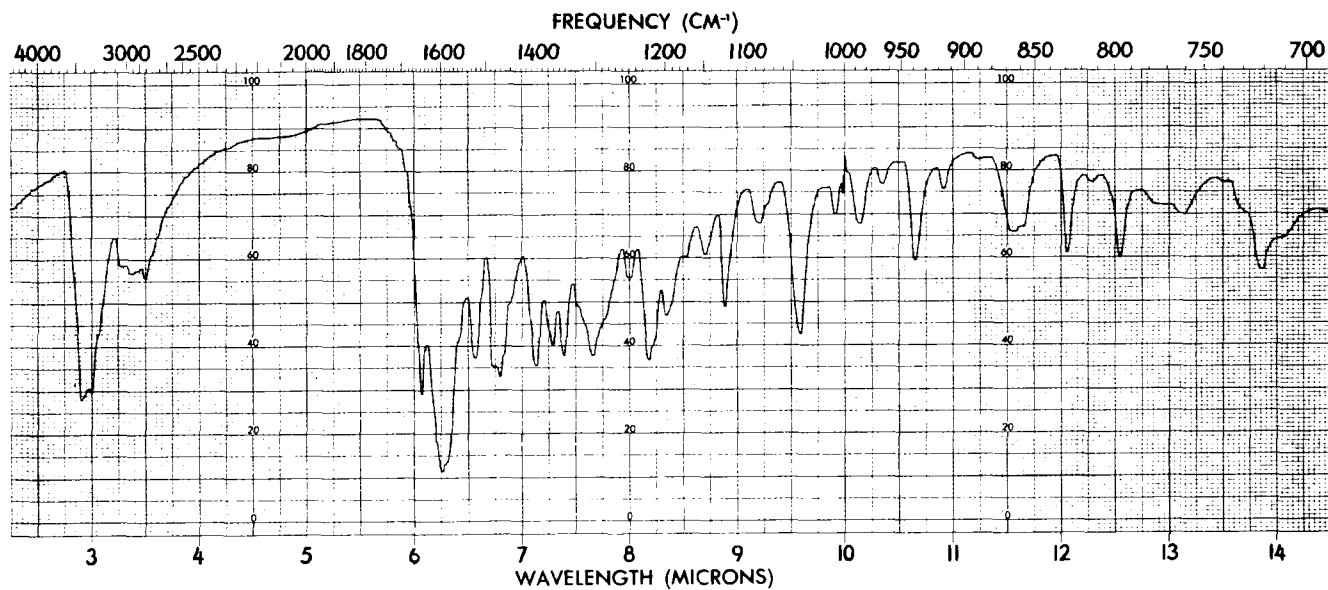
2. Physical Properties2.1 Infrared Analysis¹

The infrared spectrum of Minocycline HCl (Lederle House Standard No. 7516B-172) is presented in Figure 1.

In a multi-functional molecule like Minocycline HCl, most maxima represent a composite envelope of overlapping absorption peaks. In these cases it is not possible to uniquely

FIGURE 1

Infrared Spectrum of Minocycline HCl.2H₂O in KBr Pellet: Instrument: Perkin-Elmer 21



assign maxima. Thus, the maximum at about $2.9\ \mu$ represents the NH_2 stretching of the 2-carboxamido, together with 12 hydroxy. The remainder of the broad absorption up to $5.0\ \mu$ is composed of the hydrogen bonded phenolic and enolic hydroxy groups plus the hydrogen atom on the protonated dimethylamino group. The maxima at $6.07\ \mu$ is the carbonyl of the 2-carboxamido group, but the broad maxima centered at about $6.25\ \mu$ is a composite of conjugated hydrogen bonded ketones, plus the conjugated double bond systems present in this molecule. The maxima at about 7.7 is a composite of the strongly hydrogen bonded phenolic and enolic hydroxyl groups plus a contribution from the 2-carboxamido group and the maxima at about $8.2\ \mu$ is composed of relatively unbonded phenolic hydroxy groups.

2.2 Nuclear Magnetic Resonance Spectrum¹

The NMR spectrum, Figure 2, in hexadeuterodimethylsulfoxide containing tetramethyl silane as internal standard is a single scan on a HA-100D Varian Spectrometer. The spectral assignments of Minocycline hydrochloride are shown in Table I.

TABLE I

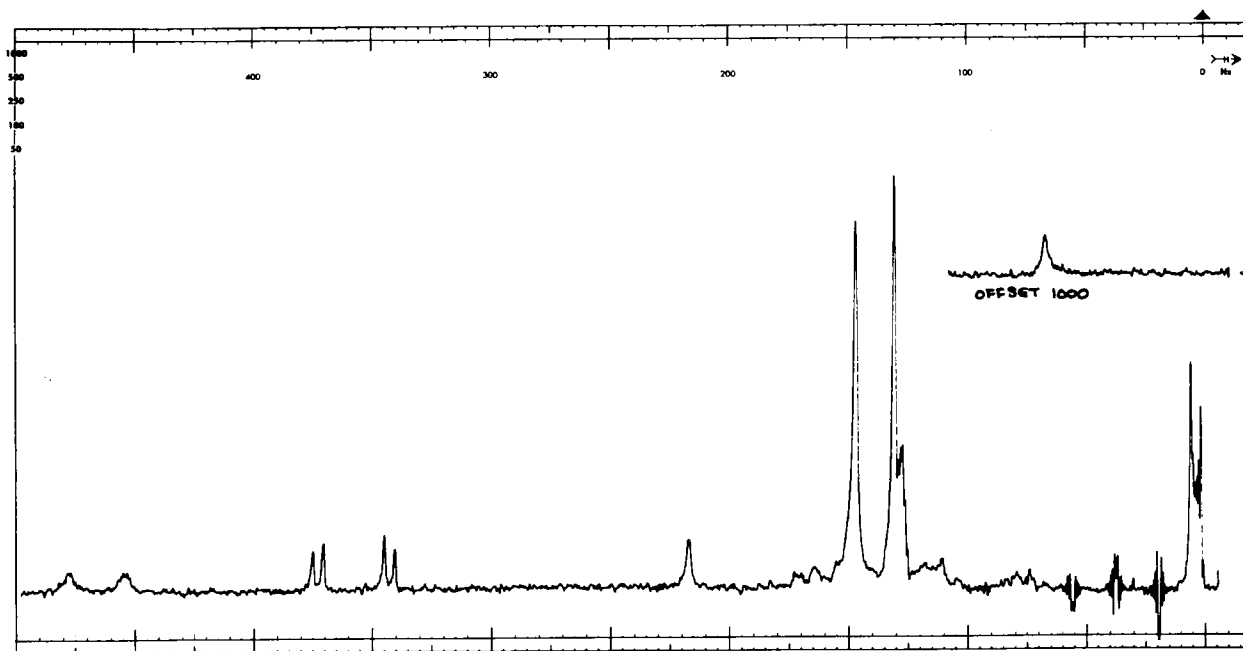
NMR Spectral Assignments of Minocycline Hydrochloride

<u>Protons at</u>	<u>Chemical Shifts (Δ)</u>		
NMe_2 (at C_4)	2.60		s
NMe_2 (at C_7)	2.94		s
C_4	4.34		s
C_8	7.41		d; $J_{8,9} = 8$
C_9	6.83		d; $J_{8,9} = 8$
$\text{C} - \text{NH}_2$	9.05	9.53	(2 broad singlets)
O			
$\text{C}_{10} - \text{OH}$	11.30		

s = singlet; d = doublet; J = coupling constant in Hz

FIGURE 2

NMR Spectrum of Minocycline HCl.2H₂O in Hexadeuteriodimethylsulfoxide Containing Tetramethylsilane as Internal Standard. Instrument: HA-100D



2.3 Ultraviolet Spectrum

Martell et al² in 1967 determined the ultra-violet properties of Minocycline. They reported -

in 0.1N HCL	λ max	352 nm (log ϵ 4.16)
		263 nm (log ϵ 4.23)
in 0.1N NaOH	λ max	380 nm (log ϵ 4.30)
		243 nm (log ϵ 4.38)

2.4 Mass Spectrum¹

The mass spectrum of Minocycline hydrochloride was run on an AEI MS-9 mass spectrometer and is shown in Figure 3. At temperatures close to the melting point the salt decomposes to the free base and HCl, and the mass spectrum is a composite of both compounds. The molecular ion of Minocycline is fairly strong and is observed at m/e 457, consistent with the elemental composition $C_{23}H_{27}N_3O_7$. Loss of NH_3 , NH_3 and $(CH_3)_2NH$, and $C_4H_3NO_3$ from the molecular ion affords ions at m/e 440, 395 and 344 respectively. A complete listing of the elemental composition of the major ions in the mass spectrum of Minocycline is available from Dr. R. T. Hargreaves, Lederle Laboratories.

2.5 Optical Rotation

The following rotation was determined¹ for Minocycline $HCl \cdot 2H_2O$ in 0.1N HCl:

$$[\alpha]_D^{25} - 166^\circ, \text{ conc.} = 0.524$$

2.6 Thermogravimetric Analysis⁷ indicates that Minocycline hydrochloride loses its water of hydration between 75° and 150° and begins to decompose at about 175°.

2.7 Differential Thermal Analysis⁷ curves for Minocycline hydrochloride exhibit one melting and/or decomposition endotherm at 217°.

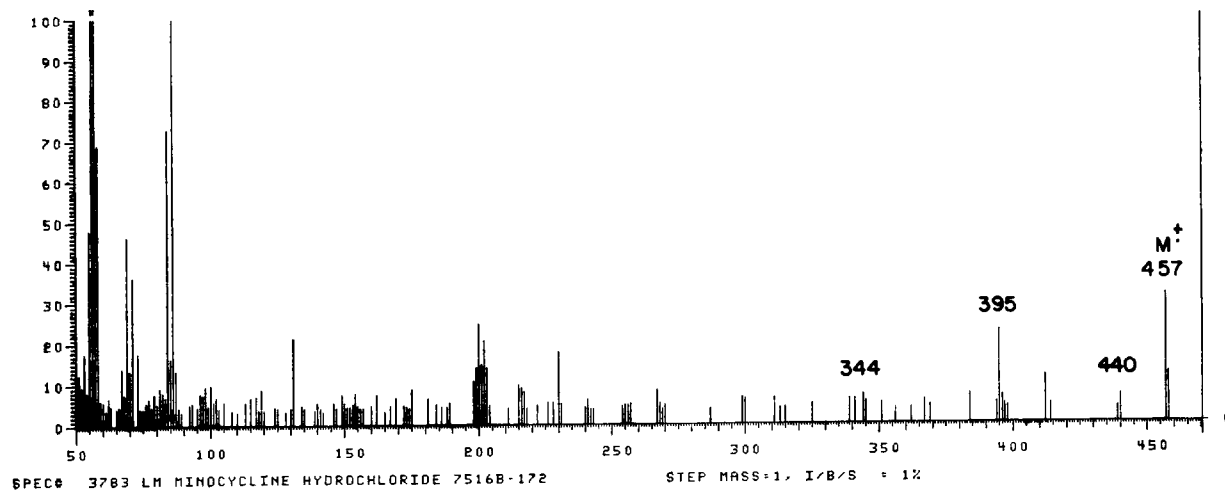
2.8 Solubility

Barringer et al³ in a monograph on Minocycline accumulated data related to unusual in vitro and in vivo properties of Minocycline and compared them to other tetracyclines antibiotics.

The solubility of tetracyclines is a complex

FIGURE 3

Low Resolution Mass Spectrum of Minocycline HCl.2H₂O. Instrument AEI MS-9



phenomenon. There are 16 possible ionic microstructures for Minocycline. Thus, the observed solubility is generally not that of a single entity but represents the sum of the total of two or more species in a solution at a given pH value. Minocycline, unlike other antibiotics, contains two amino groups which are responsible for hundred-fold solubility of Minocycline neutral in water over that of tetracycline. The solubility of Minocycline monohydrochloride dihydrate in various solvents and of Minocycline neutral in water are given in Table II and Table III respectively.

TABLE II
Aqueous Solubility of Minocycline at 25°C.

		<u>mg/ml</u>
Neutral	pH 6.7	52
Hydrochloride	pH 3.9	15
Dihydrochloride	pH 0.8	>500

TABLE III
Solubility of Minocycline Hydrochloride .2H₂O in
Various Solvents at 25°C³

<u>Solvent</u>	<u>mg/ml</u>	<u>% w/v</u>
Hexane	0.004	0.0004
Benzene	0.02	0.002
Chloroform	0.13	0.013
Ethyl Acetate	0.3	0.03
Methyl Ethyl Ketone	0.4	0.04
1-Octanol	0.5	0.05
Acetone	0.6	0.06
Dioxane	0.7	0.07
1-Butanol	4.4	0.44
2-Propanol	7	0.7
Methanol	14	1.4
Water	16	1.6
Abs. Ethanol	42	4.2

2.9 Partitioning Data

Literature values according to Colaizzi and Klink⁴ for the apparent partition coefficients of Minocycline in a water: n-octanol system at various pH values are reported in Table IV. The optimum pH value for transfer into the organic phase is about 6.6 at which pH the neutral zwitterionic form is predominant and also coincides with the isoelectric point of Minocycline.

TABLE IV

Apparent Partition Coefficients (Octanol/Aqueous Buffer) of
Minocycline Hydrochloride

pH	pH	pH	pH	pH
2.1	3.9	5.6	6.6	8.5
0	0.051	1.11	1.48	0.36

2.10 Crystal Properties

The X-Ray powder diffraction pattern of Minocycline hydrochloride is shown in Table V.

TABLE V

Powder X-Ray Diffraction Pattern of Minocycline HCl⁵

<u>d (Å)*</u>	<u>I/Io**</u>
12.0	0.15
7.05	1.00
6.60	0.04
5.70	0.08
5.20	0.07
4.95	0.09
4.73	0.09
4.45	0.01
4.28	0.06
4.00	0.04
3.82	0.15
3.68	0.50
3.56	0.45
3.43	0.02
3.26	0.40
3.03	0.04
2.86	0.05
2.73	0.02
2.67	0.02
2.60	0.01
2.44	0.06
2.31	0.02
2.25	0.02
2.13	0.02
2.06	0.01
1.96	0.01
1.91	0.01
1.85	0.03
1.72	0.02
1.52	0.01
1.20	0.02

* d = (interplanar distance) $\frac{n \lambda}{2 \sin \theta}$, $\lambda = 1.539 \text{Å}$

** Based on highest intensity of 1.00
Radiation: $K\alpha_1$, and $K\alpha_2$ Copper

3. Synthesis

Previous synthesis of Minocycline was achieved by a sequence of reactions based on nitration of 6-demethyl-6-deoxytetracycline². In this synthesis two isomers (7 and 9 nitro) were formed. Removal of undesirable 9-nitro isomer involved tedious procedures. Lately, L. Bernardi and associates⁶ were able to block position 9 with a tertiary butyl group and thus simplify the reaction and improve the yields. The reaction scheme of this new synthesis is given in Figure 4.

6-demethyl-6-deoxytetracycline (I) was alkylated to give (II) with excess of tertiary butyl alcohol and methane sulfonic acid. By adding four equivalents of KNO_2 , compound (III) was obtained in 76% yield based on (I). Intermediate compound (III) was catalytically reduced over PtO_2 to give 7-amino-9-tertiary butyl-6-demethyl-6-deoxytetracycline (IV) which was then reductively methylated to (V). The last step involved the removal of the tertiary butyl group from position 9. This was accomplished by using trifluoromethane sulfonic acid with 63% yield.

4. Stability, Isomerization, Degradation

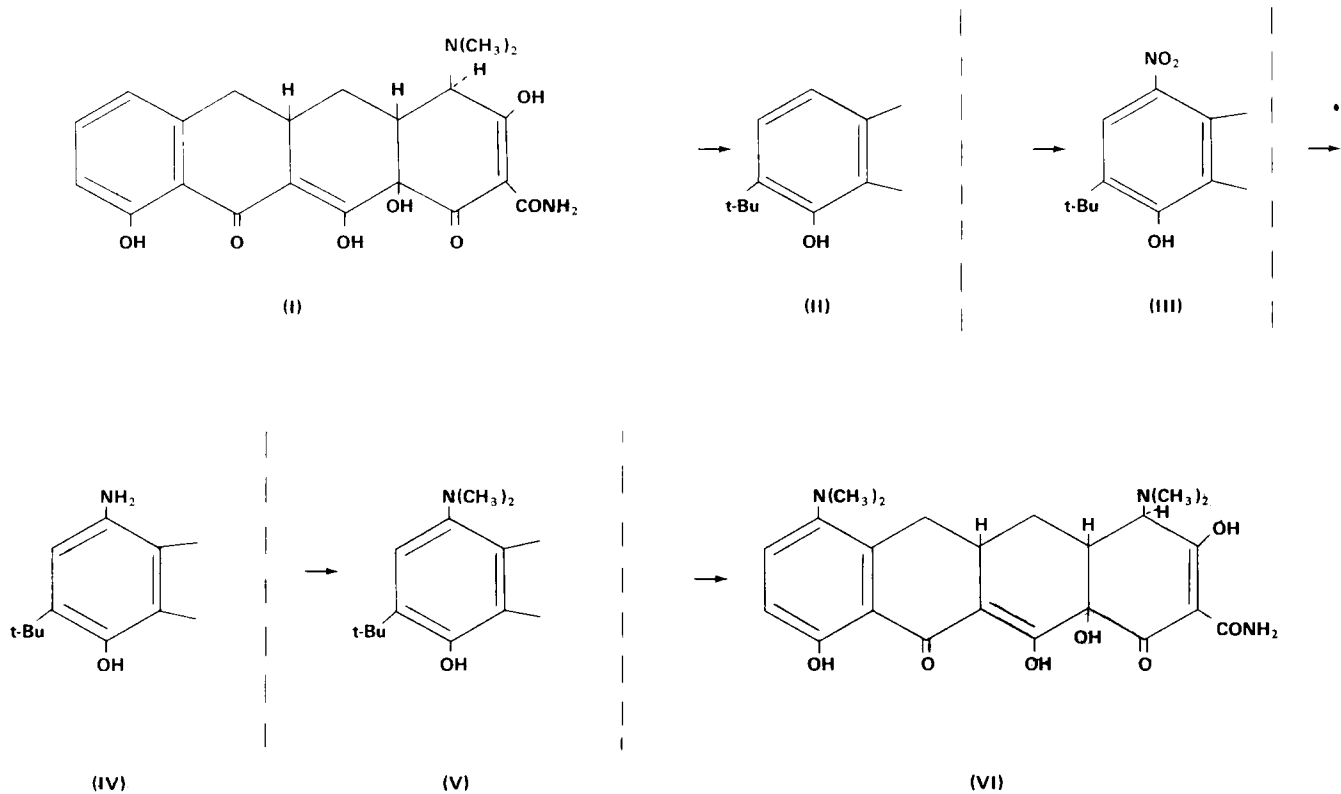
In the dry-powder state the Minocycline, like other tetracyclines, is stable at least 3-4 years when stored at room temperature (25°C). Minocycline, lacking hydroxyl groups at both C_5 and C_6 does not form the anhydro, iso, or epi compounds, which are the common degradation compounds formed from other tetracycline antibiotics. However, it readily undergoes both 4-epimerization and oxidative degradation. Since the D ring of Minocycline is a substituted p-amino-phenol, it is more susceptible to oxidation than other tetracyclines.

Stability data for solutions of Minocycline at various pH values are summarized in Table VI. Minocycline solutions at pH 4.2 and 5.2 retained 90% of their initial potency for 1 week at room temperature. These solutions were more stable than any other tetracycline antibiotic solution studied.

However, none of the tetracycline antibiotics are stable enough to permit the preparation of a preconstituted aqueous solution as a practical dosage form.

The additional amino group in Minocycline, besides contributing to increased solubility of Minocycline neutral in water, is also responsible for differences in physico-chemical

FIGURE 4.



and physiological properties. The isoelectric point of Minocycline is a full pH unit higher (pH 6.4) than that of most other tetracycline antibiotics (pH ca. 5.5) and consequently has a potential therapeutic significance. This property accounts for its greater partitioning character into lipoid material at essentially neutral pH, including thyroid, brain and fat tissue.

TABLE VI
Minocycline Solution Stability Data % Initial
Activity Retained

pH	<u>Days Stored at 25°C</u>										
	0.5	1	1.5	2	3	4	7	8	9	11	14
1.85	96	94	91	22							
2.5	97	95	93	81							
4.2		99		96	98	95	90	91	90	87	84
5.2		98		98	98	96	92	89	85	81	72
6.2		98		95	93	89	76	72	64	53	37

5. Pharmacodynamic Studies

R. C. Kelly and Associates⁸ found that the maximum serum concentration of Minocycline was attained by the first sampling at 1 hour and that serum half life after oral administration of Minocycline was 16 hours.

Minocycline showed excellent tissue penetration due to its higher zwitterionic form which is predominant at pH 6.6, approximately one pH unit higher than for other tetracyclines. An advantage for this highly lipophylic tetracycline has been postulated in terms of therapeutic efficacy, i.e. a rapid and high concentration of antibiotic where recorded. Okubo and associates⁹ established that in rats after a single oral dose, concentrations in all tissue studies were higher than in blood.

When the Minocycline was administered to patients before surgery, a similar high tissue-blood ratio was found after

the organ was removed. The highest accumulation of Minocycline was found in gallbladder, thyroid, duodenum and liver.

Minocycline is metabolized to inactive substances to a greater extent than other known tetracyclines.

6. Methods of Analysis

6.1 Elemental Analysis for $C_{23}H_{27}N_3O_7HCl \cdot 2H_2O$

<u>Element</u>	<u>% Theory</u>	<u>Reported</u>
		Ref. ²
C	52.12	52.12
H	6.09	6.19
N	7.93	7.79
Cl	6.69	6.72

6.2 Chromatographic Analysis

6.21 Thin Layer Chromatographic Analysis

Separation and quantitative determination of Minocycline in the presence of related minor components was achieved on diatomaceous earth, used as supporting phase. Plates were prepared by spreading into a thin layer a mixture of diatomaceous earth, pH 6 EDTA buffer, polyethylene glycol 400 and glycerin. Plates were developed with a solvent consisting of a mixture of pH 6 EDTA buffer and ethyl acetate-cyclohexane (9:2). This system was previously used by P. P. Ascione¹⁰ in a separation of other tetracyclines by thin layer chromatography. The R_f of Minocycline in this system was approximately 0.2. By rechromatography in the same system the Minocycline spot can be moved half way on the plate, thus giving complete separation from the related compounds.

6.22 Column Chromatographic Analysis

Minocycline and related impurities were separated on an acid-solvent washed diatomaceous earth column.^{11,12} Supporting phase was prepared by mixing the diatomaceous earth with 5% v/v polyethylene glycol 400 (PEG-400)-glycerine mixture in 0.1M EDTA pH 6 buffer. Minocycline and related compounds were eluted with stepwise increasing polarity of the chloroform-cyclohexane mixture and determined spectrophotometrically at 358 nm. 98-102% recovery of the total spectral value of the charge was obtained.

6.3 Direct Spectrophotometric Analysis

U. V. Absorption maximum of Minocycline at 358 nm has been extensively used for assay purposes, especially for reading of column eluates. The concentration of 16 micrograms per ml was used in acidified methanol-chloroform solution.¹¹

Minocycline HCl has a distinct infrared spectrum which can be used in qualitative and quantitative analysis.

A linear concentration - absorption relationship was achieved by Ace and Jaffe,¹³ using pH 6.5 buffer in an extraction of Minocycline. The fluorescence of the final product was read at an excitation wavelength of 380 nm and an emission wavelength of 480 nm using a filter colorimeter.

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NYSTATIN

Gerd W. Michel

TABLE OF CONTENTS

1. DESCRIPTION

- 1.1 Name, Formula, Molecular Weight, Elemental Composition
- 1.2 Appearance, Color, Odor
- 1.3 Standards and Regulatory Status

2. PHYSICAL PROPERTIES

- 2.1 Crystal Properties
 - 2.1.1 Optical Crystallographic Properties
 - 2.1.2 X-Ray Powder Diffraction
- 2.2 Infrared Spectrum
- 2.3 Nuclear Magnetic Resonance Spectrum
- 2.4 Ultraviolet Spectrum
- 2.5 Fluorescence Spectrum
- 2.6 Mass Spectrum
- 2.7 Optical Rotation
- 2.8 Optical Rotatory Dispersion
- 2.9 Melting Range
- 2.10 Differential Thermal Analysis
- 2.11 Thermogravimetric Analysis
- 2.12 Solubility
- 2.13 Countercurrent Distribution
- 2.14 Ionization Constants
- 2.15 Aggregation
- 2.16 Polarography

3. BIOSYNTHESIS

4. METHODS OF MANUFACTURE

- 4.1 Historical
- 4.2 Microbiological Processes
- 4.3 Isolation and Purification Processes

TABLE OF CONTENTS (Cont'd)

5. STABILITY - DEGRADATION

5.1 Dry Thermal Degradation

- 5.1.1 Stability of Amorphous Product
- 5.1.2 Stability of Crystalline Product
- 5.1.3 Stability of Solid Dosage Forms
- 5.1.4 Stability of Ointment Formulations

5.2 Stability in Solution

5.3 Stability under Radiation

5.4 Microbial Degradation

5.5 Stabilization

6. METHODS OF ANALYSIS

6.1 Elemental Analysis

6.2 Neutralization Equivalents

6.3 Identification Tests

6.4 Color Reactions

6.5 Direct Spectrophotometric Analysis

6.5.1 Fermentation Liquids and Products

6.5.2 Pharmaceutical Preparations

6.5.3 Other Applications

6.6 Colorimetric Analysis

6.7 Chromatographic Analysis

6.7.1 Paper Chromatography

6.7.2 Thin-Layer Chromatography

6.7.3 Gas-Liquid Chromatography

6.7.4 High Performance Liquid Chromatography

6.8 Electrophoretic Analysis

6.9 Polarographic Analysis

6.10 Titrimetric Analysis

6.11 Microbiological Methods

7. REFERENCES

8. ACKNOWLEDGMENT

1. DESCRIPTION

1.1 Name, Formula, Molecular Weight, Elemental Composition

Nystatin is a prominent member of a relatively large and varied group of structurally related, highly unsaturated antifungal antibiotics produced by various strains of streptomycete species of microorganisms¹⁻⁷. Based on their chemical structure - and to distinguish them from numerous other antibiotics which also have antifungal properties^{8,9} - this group of important therapeutic agents is commonly referred to as the polyene macrolide antifungal antibiotics. All members within this class of antibiotic agents have in common (a) a macrocyclic ring of carbon atoms closed by lactonization, and (b) the presence of a series of conjugated carbon double bonds.

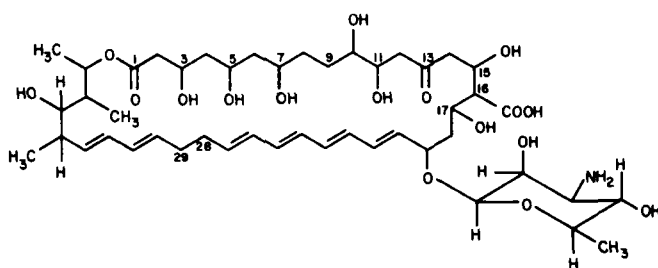
The latter grouping represents the chemically most characteristic feature of polyene macrolides and serves to further classify this group of natural products into tri-, tetra-, penta-, hexa- and heptaenes, according to the type of conjugated chromophore present in the molecule^{2,10-15}.

Attempts at complete tabulation of all presently known polyene antibiotics within this class have been published in several comprehensive review articles^{4,12,13,16-27}.

Following the above nomenclature, nystatin may be chemically classified as a tetraene macrolide antibiotic. Isolated in 1950 by Hazen and Brown²⁸⁻³¹ of the Division of Laboratories and Research, New York State Department of Health, Albany, N.Y., it was the first of the polyene macrolides to be discovered and is since produced biosynthetically on large scale by fermentation with strains of Streptomyces noursei^{32,33}, S. albulus³⁴⁻³⁶ and S. aureus^{3,6,32,34}. Initially called fungicidin^{28,29,32}, it was later given the name nystatin (N.Y. State-in)^{4,32}, but is also listed under several other proprietary synonyms^{3,37-40}: Moronal, Mycomycin, Mycostatin, Nilstat, Nitacin, Nystan and Stamicin. The designation most commonly used in the chemical, pharmaceutical and medical reference literature^{37,39-42}, including Chemical Abstracts, is nystatin.

As is true for many polyene macrolide antibiotics, a complete and satisfactory chemical characterization of nystatin with respect to its precise molecular structure, stereochemistry and absolute configuration is still outstanding,

despite extensive efforts in a number of laboratories^{32,43-60}. Early degradation studies by several investigators⁴⁹⁻⁶⁰ established the antibiotic to be a macrocyclic C₄₁-polyene lactone linked glycosidically to the pyranose form of the amino sugar mycosamine (3-amino-3,6-dideoxy-D-mannose)⁴³⁻⁴⁸. While the structure of the aglycone portion of the molecule (nystatinolide)⁴⁶, containing a diene and tetraene chromophore, has been deduced from the isolation of degradation products, Chong and Rickards⁵⁸ have only recently provided experimental evidence, subsequently confirmed by Borowski *et al.*⁵⁹, for a glycosidic linkage of the sugar moiety to the C-19 position of the aglycone. Present knowledge therefore suggests the nystatin molecule to be identical with structure I⁵⁸⁻⁶¹, without regard to its stereochemistry.

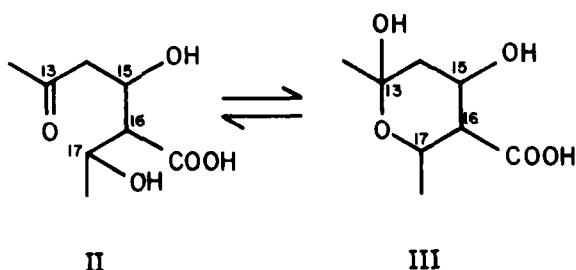


I

C₄₇H₇₅NO₁₇

Molecular Weight: 926.13

Very recent work^{58,60} has indicated that nystatin, in its crystalline state and in neutral hydroxylic solutions at ambient temperatures, may exist in the hemiketal form rather than the hydroxy-ketone structure (I) depicted above. In analogy to amphotericin B^{62,63}, a structurally related polyene macrolide whose crystalline N-iodo-acetyl derivative was found to exist as a cyclic hemiketal, a pyranoid hemiketal linkage (III) in nystatin could arise from the formation of an oxygen bridge between carbon atoms 13 and 17 of the hydroxy-ketone moiety (II), according to the following scheme:



While the available chemical evidence supports the structural characteristics of nystatin as outlined above, it should also be noted, however, that commercial nystatin products are not necessarily homogeneous compounds, but may represent mixtures of chemically closely related components^{56, 58, 59}. Shenin *et al.*⁵⁶, for instance, examined several lots of pharmaceutical grade nystatin (including the International Standard) by countercurrent distribution in a suitable solvent system and found all products to contain two chemically distinct components, A₁ and A₂, in varying proportions. In a more recent study, Porowska *et al.*^{64, 65} adopted the same technique under modified conditions to demonstrate that some commercial nystatin products may, in fact, be separated into three distinctly different constituents (designated nystatin A₁, A₂ and A₃), two of which (A₁ and A₂) are apparently identical with those characterized by Shenin *et al.*⁵⁶, while the third constituent (A₃) represents another tetraene component, also shown to be part of the polifungin-A complex produced by *Streptomyces noursei* var. *polifungini*⁶⁶⁻⁶⁹.

The lack of uniformity between individual nystatin products generated under a wide variety of possible fermentation conditions^{16, 27, 70, 71}, combined with the exceptional difficulties normally encountered in the isolation of strictly pure materials, poses unique problems in a satisfactory analytical characterization of this widely produced chemotherapeutic agent, at present. As a result, depending on the source, purity and uniformity of the examined sample, reported physico-chemical property data on nystatin can be expected to vary over a wide range and are not necessarily characteristic for the uniform, highly purified compound. Thus, for the purpose of this profile and in an attempt to overcome some of the obvious discrepancies between various literature data, a typi-

cal production lot (Squibb Research Standard #MYNM-150-RP) has been selected for characterization by the more common analytical methods, and reference is made to it whenever possible.

1.2 Appearance, Color, Odor

Nystatin is a light yellow to yellow crystalline powder with a faint, characteristically musty odor; slightly hygroscopic and light-sensitive.

1.3 Standards and Regulatory Status

The biological activity of commercial preparations of nystatin is expressed in units per mg, based on a potency of 1000 units per mg originally assigned to a batch of nystatin set aside by the FDA for reference purposes as the first primary standard. Since then, improved isolation techniques have led to the production of materials with substantially increased potencies. However, the first primary reference is still in use as a reference point in the assignment of potency values to later working standards^{40a}.

A. FDA and USP Standards

The most recently adopted FDA standard material, after collaborative assay by the National Center for Antibiotic Analysis (NCAA) and other laboratories, has been defined to contain 6088 units per mg⁷²; this material is identical with the current USP Reference Preparation of Nystatin.

B. International Standard

An international collaborative study of nine laboratories in six countries resulted in the adoption of a first International Standard (WHO Standard) for Nystatin by the World Health Organization Expert Committee on Biological Standardization in 1963⁷³. The reference material selected for this study was assayed against the USP Reference Preparation of Nystatin available at that time and was established to contain 3000 International Units (IU) per mg. Accordingly, the International Unit of Nystatin is defined as the activity in 0.000333 mg of the International Standard^{39,73}.

The methodology associated with standardization and revised outlines of the recommended standard microbiological assay procedures have been reported recently⁷⁴ and are recorded in the Code of Federal Regulations⁷⁵.

The minimum allowable potency for commercial nystatin products was reviewed by the Food and Drug Administration during 1973 and raised from 2000 units to 4400 units per mg, effective 1975⁷⁶. Official monographs for nystatin are listed in the United States Pharmacopeia XIX⁴¹ and British Pharmacopeia 1973⁴².

2. PHYSICAL PROPERTIES

2.1 Crystal Properties

2.1.1 Optical Crystallographic Properties

The following optical crystallographic constants of nystatin (without reference to crystal system and habit) have been reported^{77,78}:

Optic Sign:	+
Elongation:	-
Extinction:	parallel
Refractive Indices:	$n_{\alpha} = 1.512$
	$n_{\beta} = 1.583$
	$n_{\gamma} = 1.682$

2.1.2 X-Ray Powder Diffraction

To date, three distinctly different crystal forms of nystatin, referred to as Types A, B and C, have been observed⁷⁹. All three forms are readily identified by their characteristic X-ray powder diffraction patterns⁸⁰ (Section 2.1.2), solid-state infrared spectra⁸¹ (Section 2.2) and thermal behaviour⁸⁰ (Section 2.10). The more commonly occurring forms, Types A and B, are known to be interconvertible⁸² on changes in environmental moisture content and apparently represent crystal modifications with different degrees of hydration.

The X-ray powder diffraction data⁸⁰ for crystal forms A, B and C are given in Tables I and II, respectively, and their corresponding diffraction patterns are presented in Figure 1 (Squibb Res. Std. #MYNM-150-RP, Type A), Figure 2 (Squibb Res. Std. #MYNM-150-RP/H, Type B), and Figure 3 (Squibb Res. Std. #WSC-08982-FP, Type C), respectively.

TABLE I

X-Ray Powder Diffraction Patterns of Nystatin

<u>Type A</u>	<u>Type B</u>
Squibb Res. Std. #MYNM-150-RP (Figure 1)	Squibb Res. Std. #MYNM-150-RP/H (Figure 2)

$\frac{d(\text{\AA})^{\circ}}{}$ *	$\frac{I/I_{\circ}}{}$ **	$\frac{d(\text{\AA})^{\circ}}{}$ *	$\frac{I/I_{\circ}}{}$ **
29.0	0.34	25.0	0.27
10.5	0.32	12.6	0.40
10.1	0.15	10.8	0.15
8.70	0.22	8.60	0.26
7.80	0.11	8.00	0.17
7.10	0.22	6.90	0.46
6.34	0.85	6.43	0.36
6.0	0.29	5.90	0.47
5.31	0.37	4.98	0.48
4.76	0.17	4.52	0.92
4.45	0.85	4.20	0.70
4.32	1.00	4.00	0.69
4.08	0.78	3.77	1.00
3.79	0.39	3.13	0.17
3.23	0.16		

* d = Interplanar distance (\AA), $\frac{n\lambda}{2 \sin \theta}$

** I/I_{\circ} = Relative intensity (based on highest intensity of 1.00)

Radiation: $K_{\alpha 1}$ and $K_{\alpha 2}$ Copper

TABLE IIX-Ray Powder Diffraction Pattern of NystatinType C

Squibb Res. Std. #WSC-08982-FP
(Figure 3)

<u>$d(\text{\AA})^*$</u>	<u>I/I_0^{**}</u>
25.0	0.19
20.0	0.80
9.30	0.26
7.15	0.20
6.28	0.93
5.90	0.20
5.60	0.64
5.26	0.59
5.15	0.30
4.67	0.60
4.51	0.55
4.27	0.59
4.19	0.46
4.10	1.00
4.00	0.27
3.68	0.21
3.60	0.47

* d = Interplanar distance (\AA), $\frac{n\lambda}{2 \sin \theta}$

** I/I_0 = Relative intensity (based on highest intensity of 1.00)

Radiation: $K_{\alpha 1}$ and $K_{\alpha 2}$ Copper

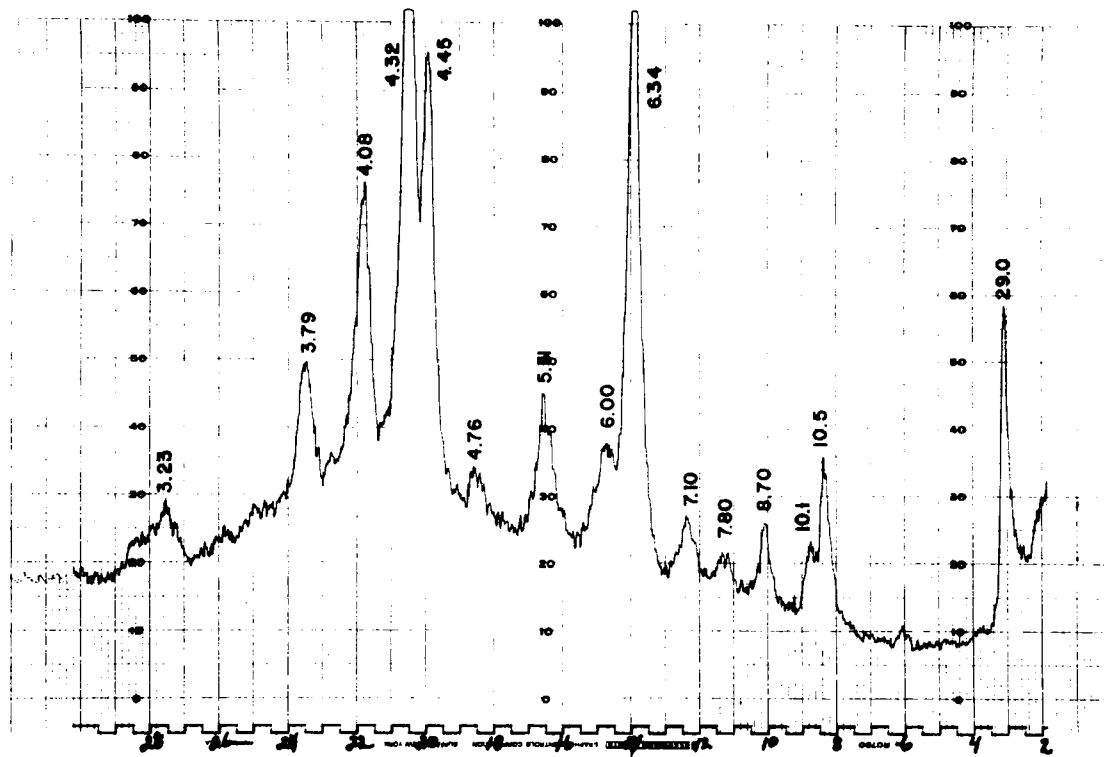


Figure 1. X-Ray Powder Diffraction Pattern of Nystatin,
Type A
(Squibb Res. Std. #MYNM-150-RP)
Instrument: Philips Norelco Diffractometer

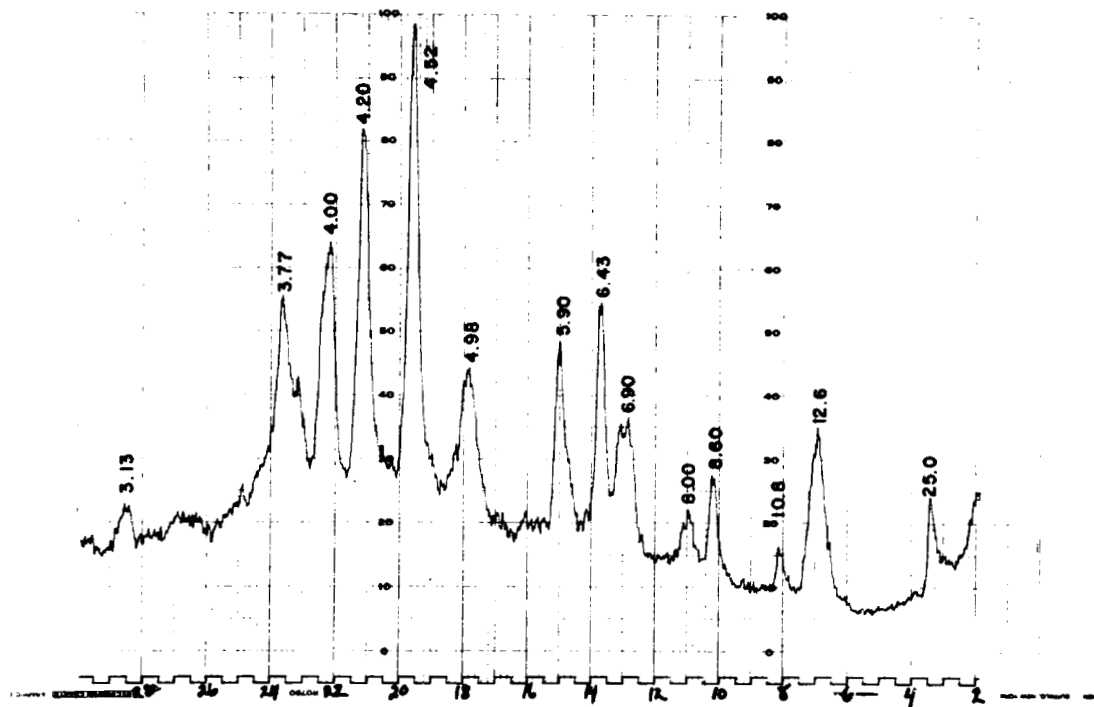


Figure 2. X-Ray Powder Diffraction Pattern of Nystatin,
Type B
(Squibb Res. Std. #MYNM-150-RP/H)
Instrument: Philips Norelco Diffractometer

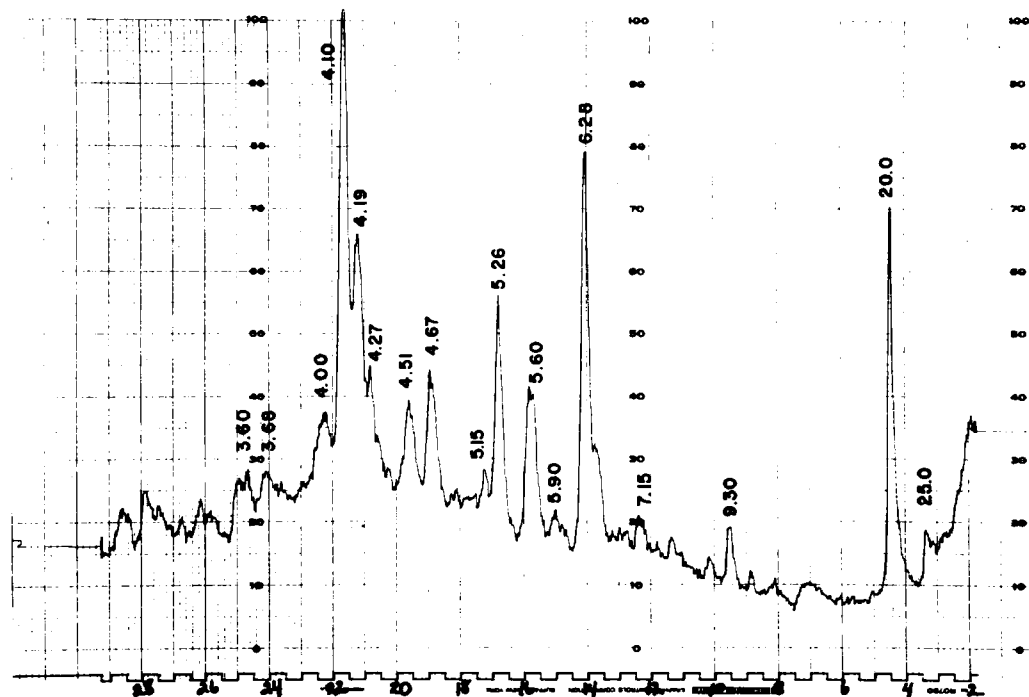


Figure 3. X-Ray Powder Diffraction Pattern of Nystatin,
Type C
(Squibb Res. Std. #WSC-08982-RP)
Instrument: Philips Norelco Diffractometer

2.2 Infrared Spectrum (IR)

The infrared absorption spectrum⁸¹ of nystatin (Squibb Res. Std. #MYNM-150-RP, Type A) as a mineral oil mull is presented in Figure 4. A spectrum of the same standard taken as a potassium bromide pellet (1.5 mg/300 mg KBr) was essentially identical to the one presented.

Tentative assignments for some characteristic infrared absorption bands^{18,53,83-85} are listed in Table III.

Table III

Infrared Spectral Assignments for Nystatin
(Squibb Res. Std. #MYNM-150-RP, Type A)

<u>Frequency (cm⁻¹)</u>	<u>Vibrational Mode^{86,87}</u>
998	CH Deformation (out-of-plane) in -CH=CH- (trans)
1065	C-OH Stretching
1375	CH ₃ Deformation (sym.)
1448	CH ₃ Deformation (asym.)
	CH ₂ Deformation
1572	Carboxylate Ion ^{18,83}
1705	Lactone (unstrained) ^{18,83}
3300-3500	NH, OH Stretching ⁸³

The IR spectrum shown in Figure 4 is in substantial agreement with spectra previously published by J.D. Dutcher *et al.*⁸³, A.O. Hayden *et al.*^{5,88} (Spectrum #85 in Hayden's compendium of spectra measured on a Perkin-Elmer Model 21 spectrophotometer with sodium chloride prism) and H. Umezawa⁸⁹.

Examination of the solid-state IR spectra (mineral oil mull) of crystal forms Type B and Type C, presented in Figures 5 and 6, resp., reveals distinct absorbance differences both between these two modifications and in their relation to the Type A form (Figure 4):

In the Type B modification, for instance, the absorption band assigned to the carboxylate ion is shifted to 1560 cm⁻¹, while the comparatively sharp band associated with the lactone carbonyl stretching vibration is observed near 1745 cm⁻¹. In addition to several other absorption changes, relative to the Type A form, in the 900-1000 cm⁻¹ and 1350-1420 cm⁻¹ regions, this form also displays a band of medium intensity near 1640 cm⁻¹.

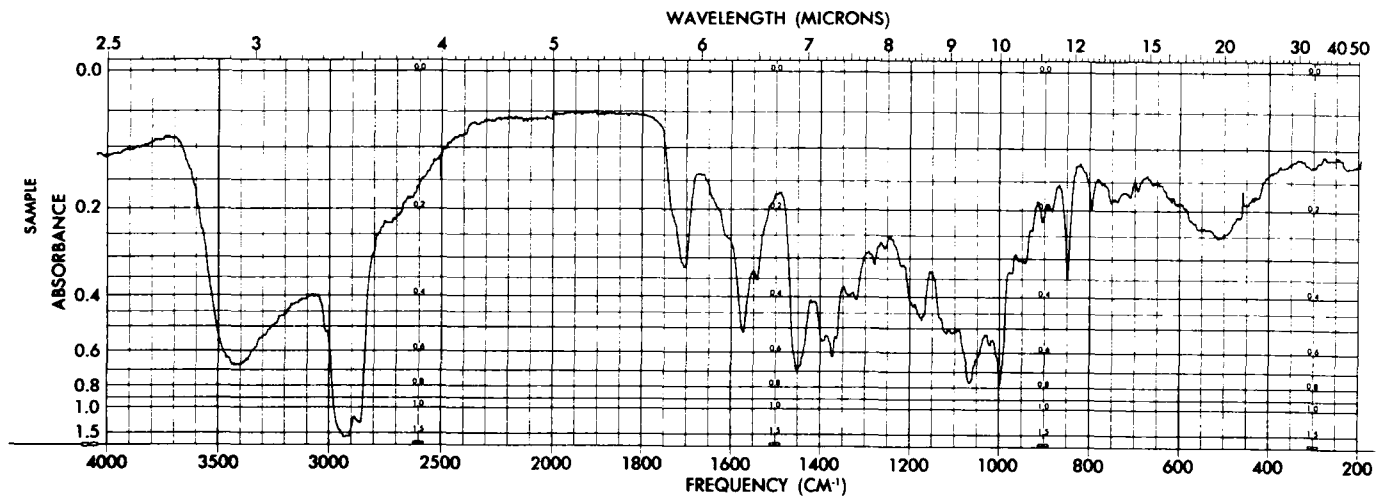


Figure 4. Infrared Spectrum of Nystatin, Type A
(Squibb Res. Std. #MYNM-150-RP)
Mineral Oil Mull
Instrument: Perkin-Elmer Model 621

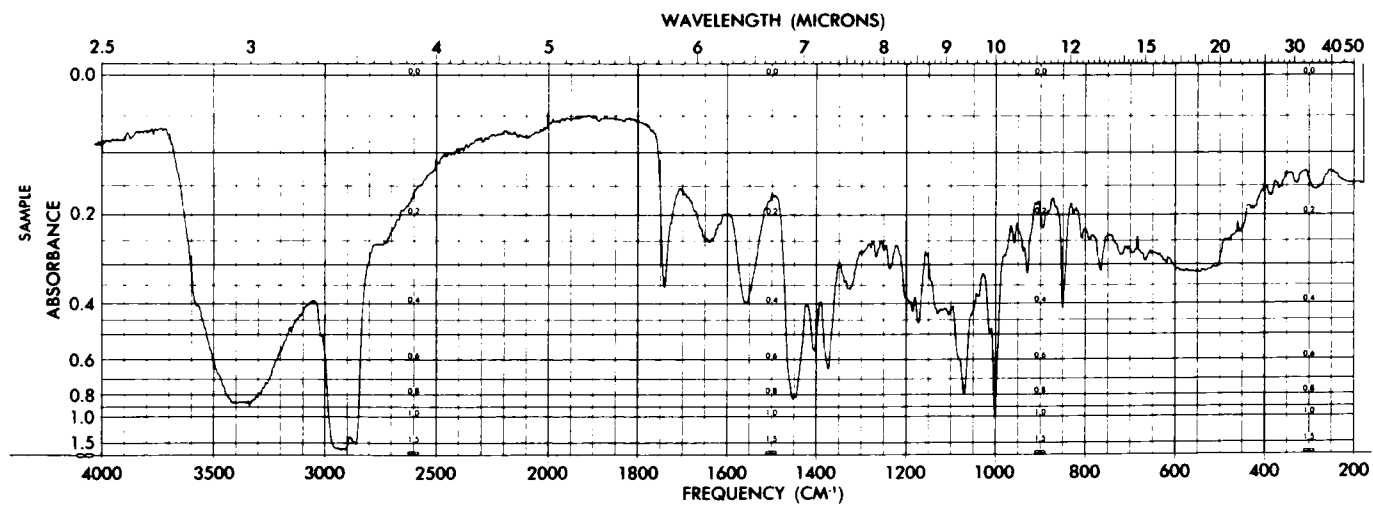


Figure 5. Infrared Spectrum of Nystatin, Type B
(Squibb Res. Std. #MYNM-150-RP/H)
Mineral Oil Mull
Instrument: Perkin-Elmer Model 621

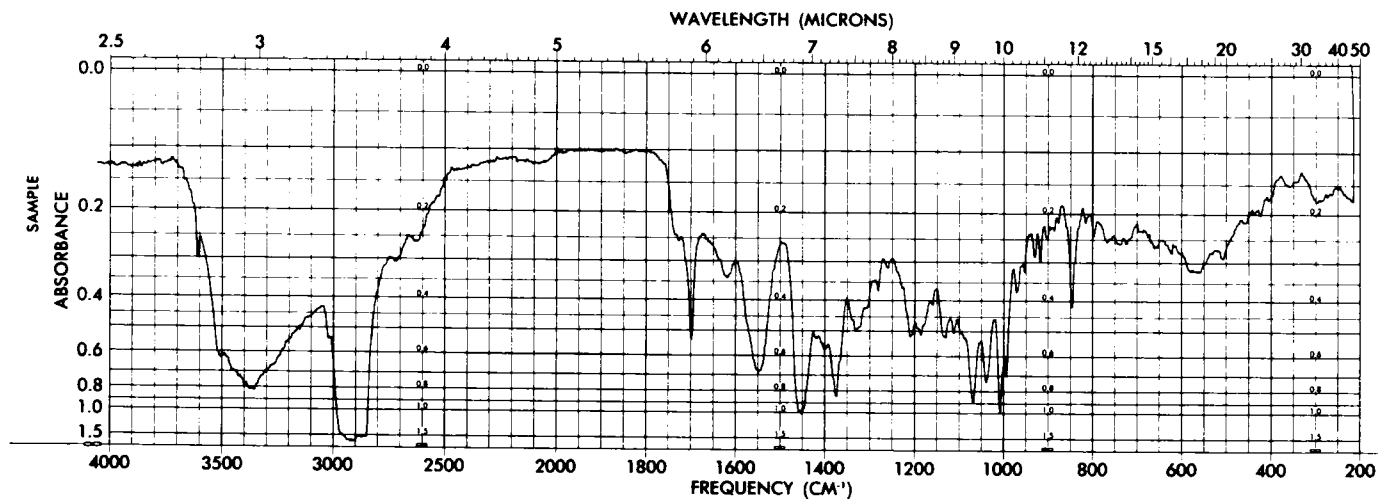


Figure 6. Infrared Spectrum of Nystatin, Type C
(Squibb Res. Std. #WSC-08982-Fp)
Mineral Oil Mull
Instrument: Perkin-Elmer Model 621

The Type C form, in contrast, is characterized by two neighboring, sharply resolved absorption bands near 990 and 1005 cm^{-1} , not present in either Type A or Type B crystal form. An additional band appears in the C-O-C stretching region near 1040 cm^{-1} , while the relatively strong, broad absorption at 1540 cm^{-1} , assigned to the ionized carboxyl group, is complemented by two weak, but definite bands at 2630 and 2700 cm^{-1} and the presence of a broad absorption near 2090 cm^{-1} , both typical for the zwitterionic structure of amino acids⁸⁶. Another strong, symmetrical band in the functional group region at 1695 cm^{-1} can be attributed to the lactone carbonyl stretching frequency. Of special diagnostic value in the identification of the Type C crystal form, however, is a sharp absorption band at 3600 cm^{-1} , absent in both Type A and Type B modifications and tentatively assigned to the "free" OH stretching mode of a cyclic hemiketal linkage (between C-13 and C-17)⁹⁰.

2.3 Nuclear Magnetic Resonance Spectrum (NMR)

The 100 MHz NMR spectrum⁹¹ of nystatin is shown in Figure 7. Proton assignments for the observed chemical shifts are tabulated below.

Table IV

NMR Spectral Assignments for Nystatin (Squibb Lot #88645)

<u>Chemical Shift</u> (ppm)	<u>Multiplicity</u>	<u>Assignment</u>
0.87 (6.0 Hz)	Doublet	Secondary Methyl Group
0.97 (6.0 Hz)	Doublet	" " "
1.10	Multiplet	" " "
1.16		" " "
1.44		Methylene Proton
1.83		" "
2.26		" "
2.78		" "
3.18		Methine Proton (-CHO-)
5.06		" " (-CHOC=O)
5.58		Olefinic Proton
5.98	Multiplet	" "
6.21		" "

In addition, broad resonance occurs at δ 3.92 (NH_2 , OH, H_2O) which is exchanged with D_2O ⁹¹.

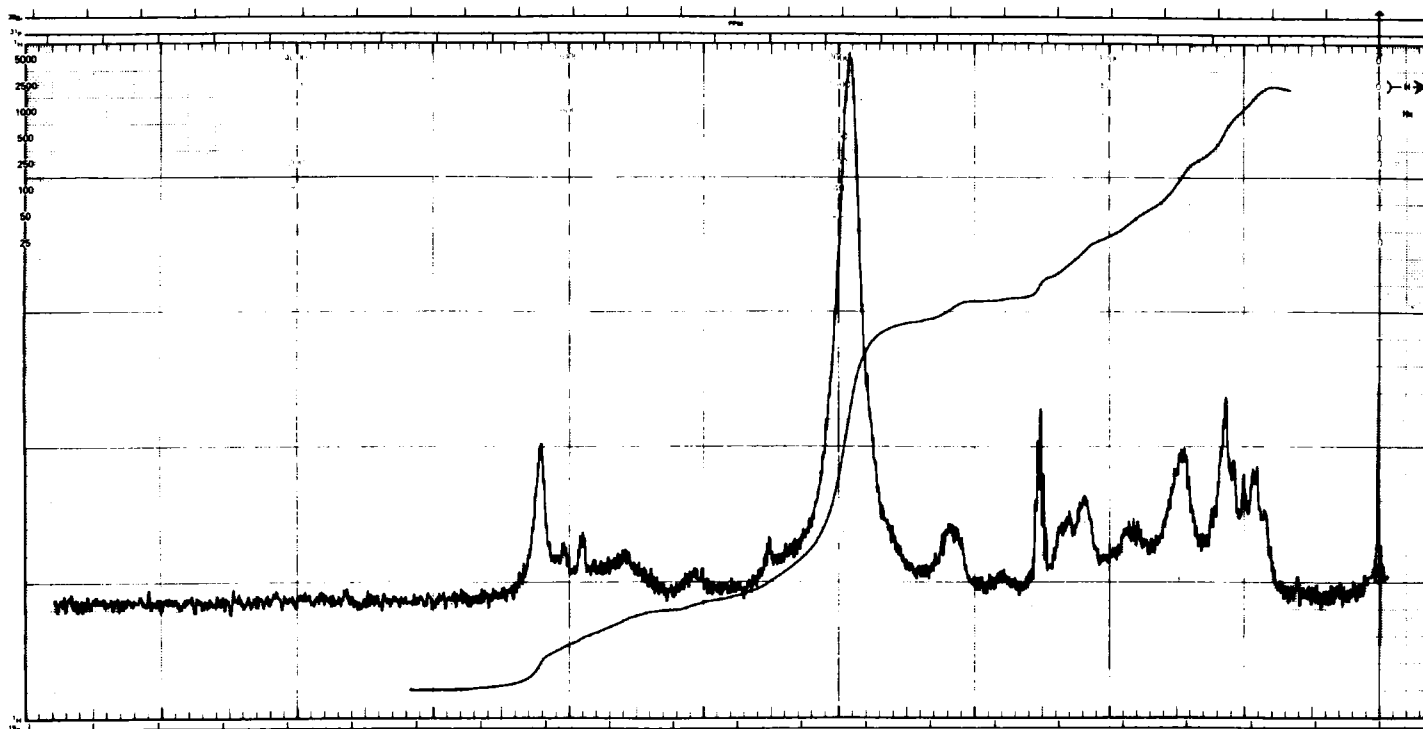


Figure 7. NMR Spectrum of Nystatin
(Squibb Lot #88645)
Solvent: DMSO- d_6
Instrument: Varian Model XL-100-15

2.4 Ultraviolet Spectrum (UV)

In agreement with the classification of nystatin as a polyene macrolide containing a conjugated tetraene and a diene chromophore, its ultraviolet spectrum exhibits three intense, very sharp absorption bands, separated by narrow valleys, in the region between 280 and 340 nm, typical for the tetraene chromophore and characteristic for several other polyene macrolide antibiotics in the same chemical category^{11,12,13,15,18a,92,93}.

The ultraviolet absorption spectrum⁹⁴ of nystatin reproduced in Figure 8 was obtained from a methanol solution of Squibb Res. Std. #MYNM-150-RP at a concentration of 1.076 mg per 100 ml of methanol. Since methanolic solutions of nystatin are known to have a limited stability, the spectrum was recorded within 10 min. after sample preparation. Under these conditions, the following three principal absorption bands were obtained:

λ_{max} nm	E (1%, 1 cm)
280 (sh)	298
291	567
304	866
318	789

These three distinct, regularly spaced peaks - characteristic for unhindered, coplanar systems of conjugation - form the main absorption bands for nystatin and are assigned to the tetraene chromophore (possibly an all-trans configuration)^{12,18a,83,95}.

A minor inflection^{5,32,83,88} is noted at 280 nm ($E_{1\%}^{1\text{cm}} = 298$), and an additional band at 231 nm of lower absorptivity ($E_{1\%}^{1\text{cm}} = 290$) has been attributed to the diene linkage (trans,trans-1,4-disubstituted)^{18a,83}.

The spectrum is in good agreement with the absorbances originally recorded for nystatin by Brown and Hazen³⁰, by Dutcher *et al.*^{32,83,95} and those documented by other investigators, as listed in Table V.

Two similar spectra of nystatin, measured as methanol solutions in the presence of 0.1% of glacial acetic acid and 0.1% of 0.1N sodium hydroxide, respectively, are listed in the collection of USP and NF reference standards compiled by

TABLE V
Ultraviolet Absorption of Nystatin

<u>Source</u>	<u>Reference</u>	<u>$\lambda\lambda_{\max}$ (nm)</u>
Bolshakova <u>et al.</u>	52	291, 304, 318
Brown and Hazen	30	291, 305, 319
Doskochilova and Gess	96	230, 292, 304.5, 318
Dutcher	95	230, 290, 305, 320
Dutcher <u>et al.</u>	32	292, 304.5, 318
Dutcher <u>et al.</u>	83	231, 292, 305, 320
Hamilton-Miller	97	292, 306, 321
Oroshnik and Mebane	18a	230, 291, 304, 318.5
Oroshnik <u>et al.</u>	12	292, 304.5, 318
Shenin <u>et al.</u>	56	230, 291, 304, 319
Umezawa	89	235, 291, 304, 319
Vining <u>et al.</u>	13	292, 304.5, 318

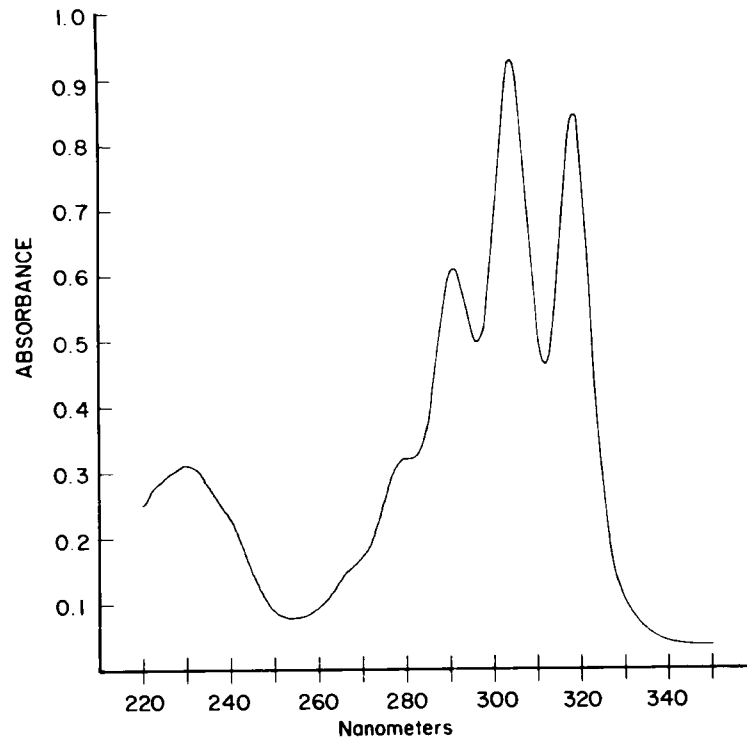


Figure 8. Ultraviolet Spectrum of Nystatin
(Squibb Res. Std. #MYNM-150-RP)
Solvent: Methanol (1.076 mg/100 ml)
Instrument: Cary 11 Spectrophotometer

Hayden et al.^{5,88}. The corresponding absorbance maxima (#85 of Hayden's compendium) are quoted as follows: 280,290,304 and 318 nm (in acidic medium), and 230,280,290 and 317 nm (in alkaline medium).

The special nature of the ultraviolet absorption spectra of polyene macrolide antibiotics and their significance in the interpretation of structural differences between closely related Streptomyces antifungal polyenes are thoroughly discussed in a review article by Oroshnik and Mebane^{18a}.

2.5 Fluorescence Spectrum

Schroeder et al.⁹⁸, utilizing a computer-centered combination spectrophotometer-spectrofluorometer system, examined the fluorescence properties of freshly prepared aqueous nystatin solutions (8.39 μ M in 0.05M citrate-phosphate buffer, pH 4, containing 0.3% dimethylsulfoxide) and observed corrected maxima for excitation and fluorescence, respectively, at 323 and 402 nm.

Similar activation and emission data are reported by Kadin⁹⁹ for dilute solutions of nystatin in a 1:1 (by vol.) methanol/water system containing approximately 5 micrograms of substrate per ml of solvent. Under these conditions, using a Perkin-Elmer Model 204 fluorescence spectrometer, excitation maxima were observed at 310 and 321 nm, with corresponding maximum fluorescence emission at 429 and 409 nm, respectively.

The excitation and emission spectra of nystatin (Squibb Lot #88645), recorded by Noone¹⁰⁰ and obtained from a methanol solution at a concentration of 10 ppm, are presented in Figure 9. Excitation at 325 nm produced emission with a maximum at 422 nm.

2.6 Mass Spectrum

The use of mass spectrometry with respect to nystatin has been limited to the determination of molecular weights and the identification of cleavage products in early structure elucidation studies^{54,57-59}, but has not been extended to investigations of the intact, underivatized molecule, most likely because of inherent problems associated with its high molecular weight and the complex, polyfunctional nature of the molecule.

Recently, however, Haegele and Desiderio¹⁰¹ examined the pertrimethylsilylated (per-TMS) derivative of nystatin and

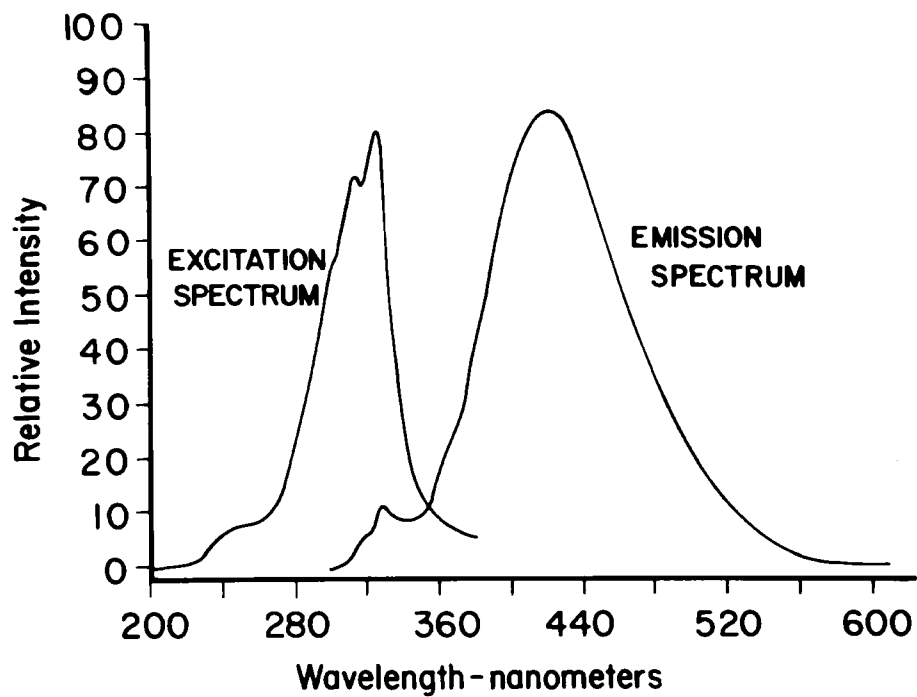


Figure 9. Fluorescence Spectra of Nystatin
(Squibb Lot #88645)
Solvent: Methanol
Instrument: Aminco-Bowman SPF

reported its complete low resolution mass spectrum, including a detailed rationalization for the genesis of the observed ion species and a proposal for the respective fragmentation pathways. The mass spectral fragmentation pattern of per-TMS nystatin is characterized by consecutive losses of MTS, TMSOH and the mycosamine moiety, with the most abundant ions in the low mass range of the spectrum arising from the amino sugar portion of the molecule. The authors¹⁰¹ conclude that the apparent driving force behind most of the fragmentation processes is to be sought in the energetically favored extension of the conjugated polyene system to a highly conjugated ion species (m/e 870) and the production of neutral molecules, facilitated by the stability of the leaving groups - TMSOH and the amino sugar moiety.

Other important features of the mass spectrum of per-TMS nystatin include:

- (a) Loss of a TMS group produces an ion cluster at m/e 1716; elimination of three molecules of TMSOH from m/e 1716 leads to the formation of ions at m/e 1626, 1536 and 1446.
- (b) Elimination of the amino sugar portion - with retention of the glycosidic oxygen by the aglycone - produces the $[M-362]^+$ ion at m/e 1427; it loses in succession eight molecules of TMSOH to form the respective ion species.
- (c) Expulsion of the neutral sugar moiety forms the $[M-379]^+$ ion at m/e 1410; the required hydrogen atom for this process is postulated to arise from C-18 to produce an ion in which the conjugation is extended. Up to six molecules of TMSOH are then eliminated from this ion to form a series of ions (m/e 1320, 1230, 1140, 1050, 960) and to produce finally the highly conjugated ion at m/e 870.
- (d) Loss of one and two molecules of TMSOH from $[M]^+$ generates ions at m/e 1699 and 1609.

The proposed fragmentation mechanisms have been corroborated by stable deuterium isotope (d_9) derivatives and were confirmed by accurate mass measurements.

For the formation of the TMS derivative, standard published procedures were followed by the authors¹⁰¹ without

modification. Low resolution mass spectra were obtained with an Atlas/Varian CH-7 mass spectrometer and high resolution spectra on a DuPont/CEC 21-110B instrument. Detailed instrumental conditions are given¹⁰¹.

2.7 Optical Rotation

Early investigators determined the specific rotation of nystatin in several solvents; their data, and those characteristic for Squibb Lot #88645 are as follows:

$[\alpha]_D^T$	T, °C	Solvent	Reference
-10°	25	AcOH (C, not specified)	18a, 32
-8°	25	AcOH (C, not specified)	83
-8°	-	AcOH (C, not specified)	95
+21°	25	Pyridine (C, not specified)	18a, 32, 83
+12°	25	DMF (C, not specified)	18a, 32
-7°	25	0.1N HCl in MeOH (C, not specified)	18a, 32

Squibb Lot #88645

+ 8.05	22.5	DMF (C = 1)	94
+21.04°	22.5	Pyridine (C = 1)	94

2.8 Optical Rotatory Dispersion (ORD)

The optical rotatory dispersion curve of nystatin (methanol solution) in the 250-450 nm region has been presented by Chong and Rickards⁶⁰; from a comparison of the ORD characteristics of the parent antibiotic with those of its dihydro- and perhydro-derivatives, the authors conclude that nystatin - in neutral hydroxylic solution at ambient temperatures - is likely to exist as a cyclic hemiketal (in analogy to amphotericin B)⁶².

2.9 Melting Range

Nystatin does not exhibit a sharp melting point. Dutcher et al. report gradual decomposition above 160°C³² and

165°C⁸³, respectively, without melting by 250°C.

Squibb Res. Std. #MYNM-150-RP, when heated on a Mettler Model FP52 microscope hot stage at a rate of 10°C/min and viewed in polarized light, shows a distinct phase transition at 165.5-168.5°C with concurrent loss of birefringence.

2.10 Differential Thermal Analysis (DTA)

The thermal properties of nystatin vary markedly with the nature of the crystal modification (Types A, B and C; see Sections 2.1.2 and 2.2), and their specific characteristics represent a useful supplementary tool in the identification of each of the three observed forms. A differential thermal analysis (DTA) study of the recognized modifications was performed by Jacobson and Valentil¹⁰² between room temperature and 250°C using a DuPont Model 900 Differential Thermal Analyzer under the following operating conditions:

Sample:	Microtube (1.6-1.8 mm)/ Air Atmosphere
Reference:	Glass Beads
Heating Rate:	15°C/min
Temperature Scale:	50°C/in.
ΔT:	1°C/in.

The respective thermograms⁹⁴, reproduced in Figure 10, show the following prominent features:

Type A (Squibb Res. Std. #MYNM-150-RP)

Single, well-defined endotherm at 169°C (corr.), corresponding to the sharp phase transition discernible under polarized light on heating of the sample on a microscope hot stage (Section 2.9). Above this temperature, rapid decomposition takes place.

Type B (Squibb Res. Std. #MYNM-150-RP/H)

Two sharp endotherms at 115°C and 171°C (both corr.).

Type C (Squibb Res. Std. #WSC-08982-FP)

Single sharp endotherm at 153°C (corr.), followed by a broad endothermal band in the 160-185°C range.

2.11 Thermogravimetric Analysis (TGA)

A thermogravimetric analysis (TGA) of samples of the

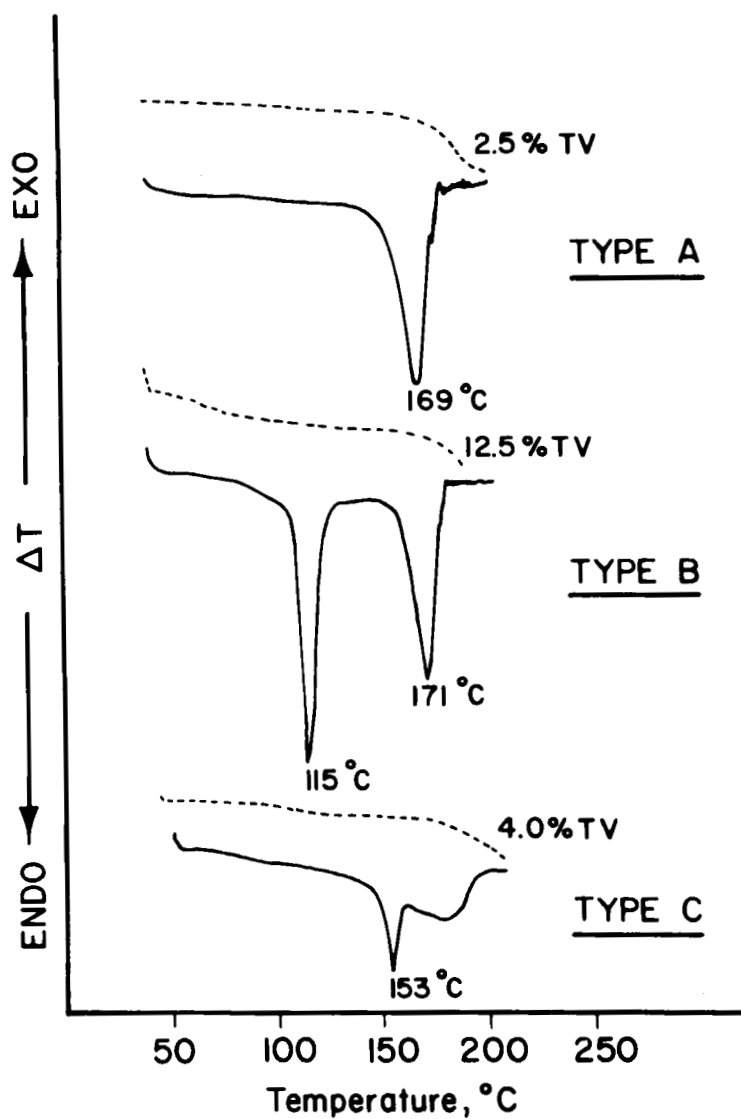


Figure 10. DTA and TGA Thermograms of Nystatin
(Types A, B and C)
Instruments;
DuPont Model 900 Differential Thermal Analyzer
DuPont Model 950 Thermogravimetric Analyzer

three identified crystal modifications of nystatin (Types A, B and C; see Sections 2.1.2 and 2.2) under a nitrogen atmosphere has been conducted¹⁰² using a DuPont Model 950 Thermogravimetric Analyzer under the following operating conditions:

Sample Atmosphere: Nitrogen Sweep (30-40 ml/min)
 Heating Rate: 15°C/min
 Temperature Scale: 50°C/in.
 Sensitivity: 2 mg/in.

The corresponding TGA curves⁹⁴, superimposed on Figure 10, indicate the following continuous weight losses for the three crystal forms:

	Weight Loss %	Temperature
Type A	2.5 ~15	up to 130°C up to 200°C
Type B	12.5 ~20	up to 130°C up to 185°C
Type C	4.0 ~12	up to 130°C up to 200°C

2.12 Solubility

Nystatin is practically insoluble at room temperature in water and common non-polar solvents, sparingly soluble in lower aliphatic alcohols, and readily soluble in formamide, N,N-dimethylformamide, dimethylsulfoxide, pyridine, ethylene glycol and propylene glycol^{29,32,37,83}. Its solubility in polar solvents is reported to be substantially increased in the presence of 10 to 20% water³².

Solutions and suspension of nystatin in water³⁷, lower alcohols, highly alkaline and acid media (e.g., glacial acetic acid, 0.05N methanolic HCl or NaOH)^{32,83,95} are rapidly inactivated soon after preparation.

As part of a comprehensive study of 18 different antibiotics completed in 1957, Weiss *et al.*^{5,103} reported the solubility of pooled commercial nystatin samples in 24 solvents at room temperature ($28 \pm 4^\circ\text{C}$). These data, together with the results of solubility determinations for Squibb Lot #88645 in several selected solvents at $24 \pm 1^\circ\text{C}$ ¹⁰⁴, are summarized in Table VI. The discrepancies between the results of

both determinations are noted and evidently result from differences in the purity and/or homogeneity of the examined samples.

Table VI
Solubility of Nystatin

<u>Solvent</u>	<u>Solubility, mg/ml</u>	
	<u>Weiss et al.¹⁰³</u> <u>[28+4°C]</u>	<u>Squibb Lot #88645¹⁰⁴</u> <u>[24+1°C]</u>
Water	4.0	0.36
Methanol	11.2	10.23
Ethanol	1.2	0.83
2-Propanol	1.2	0.23
Isoamyl Alcohol	2.4	
Cyclohexane	0.505	<0.1
Benzene	0.28	<0.1
Toluene	0.285	
Petroleum Ether	0.16	
2,2,4-Trimethylpentane	0.03	
Carbon Tetrachloride	1.23	<0.1
Ethyl Acetate	0.75	
Isoamyl Acetate	0.55	
Acetone	0.39	0.10
Methyl Ethyl Ketone	0.75	
Diethyl Ether	0.30	
1,2-Dichloroethane	0.45	
1,4-Dioxane	2.1	
Chloroform	0.48	<0.1
Carbon Disulfide	0.40	
Pyridine	>20	
Formamide	>20	
Ethylene Glycol	8.75	16.63
Benzyl Alcohol	2.65	

As part of a general study of the physical properties of nystatin intermediates isolated by mycelium extraction with lower alcohols and vacuum concentration of the resulting aqueous alcoholic extracts, Trakhtenberg *et al.*¹⁰⁵ examined the effect of changes in the water content of several solvents (acetone, methanol, ethanol and 2-propanol) on the solubility of the isolated products. While methanol-water mixtures provided maximum solubility for the antibiotic intermediates at water levels below 10 vol.%, the authors¹⁰⁵ found substantial increases in the solubility of the test products in the binary systems ethanol/water, isopropanol/water and acetone/water

with increasing water concentrations (ranging up to 50 vol.% in isopropanol). In contrast, the solubility of the examined materials in 70% aqueous methanol was determined to be only one-half of their solubility in neat methanol (8.26 mg/ml).

In a similar, later study conducted with a slightly purer, crystalline product (activity 4500 units/mg), Kleiner and Ionoval¹⁰⁶ examined the solubility of nystatin in binary mixtures of methanol, ethanol and isopropanol containing up to 50 vol.% of water and, in substance, confirmed the general observations made by Trakhtenberg *et al.*¹⁰⁵ with less pure preparations. While the solubility of nystatin in methanol was again found to have its maximum (9.2 mg/ml, $24 \pm 1^\circ\text{C}$) in the absence of water, solubilities were shown to be greatly enhanced in ethanol and isopropanol with increases in water content in both solvents. Maximum solubilities for nystatin were reported to reach 4.0 mg/ml in 75 vol.% aqueous ethanol (0.55 mg/ml in anhydrous ethanol) and 2.2 mg/ml in 70 vol.% aqueous isopropanol (0.68 mg/ml in anhydrous isopropanol) at $24 \pm 1^\circ\text{C}$, as compared to 9.2 mg/ml in anhydrous methanol at the same temperature.

Solubility profiles of nystatin for the solvent systems methanol/water and ethanol/water ($23 \pm 1^\circ\text{C}$) have been determined⁸² with Squibb Res. Std. #MYNM-150-RP, following (a) the gravimetric procedure outlined by Weiss *et al.*¹⁰³, and (b) a spectrophotometric method referred to in Section 6.5. Individual solubility data are summarized in Table VII, and the corresponding solubility curves are presented in Figures 11 and 12.

2.13 Countercurrent Distribution

In 1968, Shenin *et al.*⁵⁶ described a method for the separation of commercial nystatin preparations into two closely related components, designated A_1 and A_2 , by countercurrent distribution in an n-amyl alcohol/isoamyl alcohol/pH 5 citrate-phosphate buffer system. In particular, the selected method involved 200-transfer distributions and the isolation of the pure constituents by subsequent extraction of the upper phase with a three-fold volume of petroleum ether, followed by washing of the organic phase with water and acetone, removal of the solvent and drying of the resulting residue.

Subsequent redistribution of the individually isolated components A_1 and A_2 in the same solvent system showed the complete absence of the companion fraction originally present in the starting material, thus evidently excluding the possi-

bility that either component may be the product of partial degradation during experimentation.

Table VII

Solubility of Nystatin in MeOH/H₂O
and EtOH/H₂O Systems at 23 ± 1°C
 (Squibb Res. Std. #MYNM-150-RP)

% Solvent (vol./vol.)	MeOH/H ₂ O		EtOH/H ₂ O	
	Gravi- metric Method mg/ml	Spectro- photom. Method u/ml*)	Gravi- metric Method mg/ml	Spectro- photom. Method u/ml*)
100	9.0440	52,193	1.1240	3800
98	9.6530	54,627	1.0880	4175
96	9.8440	55,428	1.5160	5910
94	-	-	2.0520	9045
93	9.4520	53,493	-	-
92	-	-	2.3680	11,713
90	8.8080	47,957	2.3920	10,972
80	-	-	1.8560	7284
75	2.4333	11,019	-	-
70	-	-	1.7240	6403
65	-	-	1.5960	6463
60	-	-	1.5560	5956
50	0.6520	1961	1.1480	4322
40	-	-	0.7920	2528
30	-	-	0.5720	1191
25	0.3240	-	-	-
20	-	-	0.3960	450
10	-	-	0.2480	356

*) Based on a potency for Squibb Res. Std. #MYNM-150-RP of 6190 u/mg (spectrophotometric assay, Section 6.5).

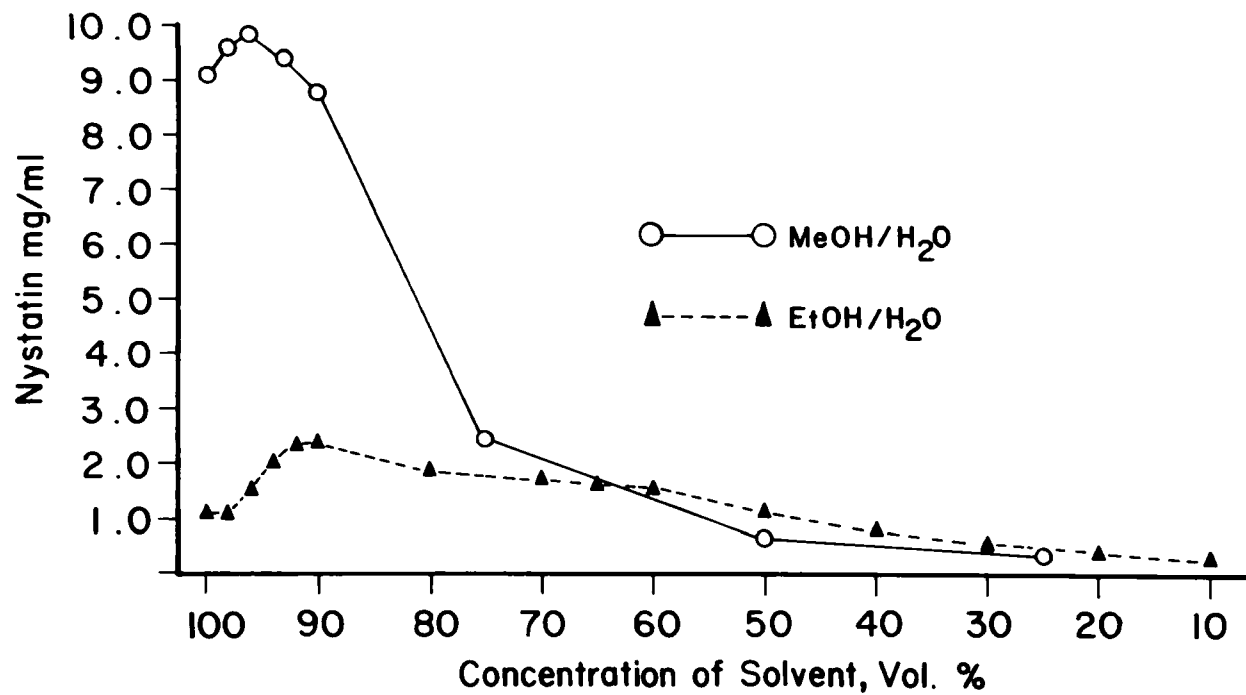


Figure 11. Solubility Profile of Nystatin
 (Squibb Res. Std. #MYNM-150-RP)
 Solvent Systems: Methanol/Water, $23 \pm 1^\circ\text{C}$
 Ethanol/Water, $23 \pm 1^\circ\text{C}$

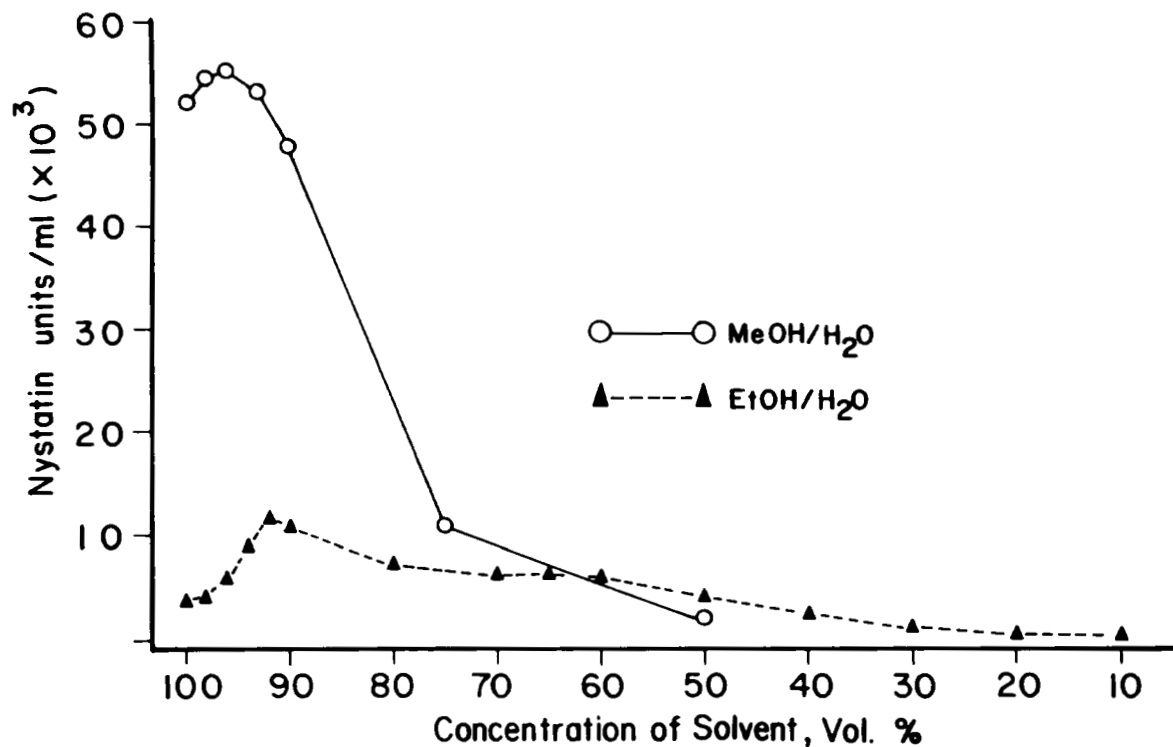


Figure 12. Solubility Profile of Nystatin
 (Squibb Res. Std. #MYNM-150-RP)
 Solvent Systems: Methanol/Water, $23 \pm 1^\circ\text{C}$
 Ethanol/Water, $23 \pm 1^\circ\text{C}$

The authors⁵⁶ examined several pharmaceutical grade products and found all of them to contain both components, although in varying ratios, depending on their origin and/or their degree of purity, but generally established the A₁-component (distrib. coefficient 4.6) to be present in much larger quantities than the A₂-component (distrib. coefficient 16.8).

Although both fractions apparently represent distinct chemical species, they nevertheless exhibit a number of closely related features, including essentially identical IR spectra, similar UV spectra characteristic of a tetraene chromophore and, when subjected to acid hydrolysis, both constituents yield mycosamine as one of the reaction products. Moreover, as freshly generated products, both components are said to exhibit effectively equal bioactivities. However, a marked difference between both components was found in their relative stabilities as determined by an "express" method not further described in detail. While, under these conditions, the A₂-component was found to remain stable, component A₁ lost approx. 50% of its initial bioactivity.

Other investigators^{107,108} have reexamined the findings reported by Shenin et al. with various samples of pharmaceutical grade nystatin and - despite the lack of adequate experimental details in the original publication⁵⁶ - were able to confirm the presence of two or more constituents in all examined nystatin products.

Recently, Porowska et al.^{64,65} adopted a counter-current distribution technique to establish the close chemical relationship between nystatin and polifungin (produced by Streptomyces noursei var. polifungini, ATCC 21581), while also being able to demonstrate that both tetraene antibiotics are not homogeneous entities but, in fact, represent complexes of up to four biologically active main components. During the course of this investigation, samples from several lots of pharmaceutical grade nystatin were shown to be separable by consecutive countercurrent distribution from two different solvent systems (methanol/chloroform/pH 8.2 borate buffer and methanol/chloroform/1% aq. NaCl soln.; 400 transfers) into three closely but chemically distinct constituents designated as nystatin A₁ (main component), A₂ and A₃. On comparison to similar fractions isolated concurrently from the polifungin complex, all three pure components separated from nystatin were also found to be common to polifungin. Moreover, the evidence presented suggests that two of the constituents derived from the nystatin complex, namely A₁ and A₂, are evidently identical with those characterized by Shenin et al.⁵⁶,

while the third component (A_3) represents a newly isolated bioactive constituent.

Based on the evidence at hand, as supported by TLC and bioautographic comparisons, the authors^{64,65} conclude that all three nystatin constituents are identical with those contained in the polifungin-A complex, while the fourth tetraene component separated from the polifungin complex, designated polifungin B, is apparently the only main constituent differentiating both nystatin and polifungin complexes from each other.

2.14 Ionization Constants

Nystatin is an amphoteric compound with two ionizing groups, namely a carboxyl and an amino function. Ray-Johnson¹⁰⁹ determined the ionization constants of nystatin in a mixture of N,N-dimethylformamide/water (50:50) by direct titration and - following the general procedure of Albert and Serjeant¹¹⁰ - calculated the following pK_a values from the titration curves:

$$\begin{aligned}pK_1 \text{ (proton gained)} &= 5.12 \\pK_2 \text{ (proton lost)} &= 8.89\end{aligned}$$

Recently, Valenti¹⁹⁵ determined the ionization constants and the isoelectric point of nystatin in a ternary solvent system composed of methanol, 2-methoxyethanol and water by potentiometric titration and established the following apparent pK_a values from the respective equilibrium constants:

$$\begin{aligned}pK_1 &= 5.72 \\pK_2 &= 8.64\end{aligned}$$

The isoelectric point for nystatin in this system, calculated from the average of pK_1 and pK_2 , was found to be at pH 7.18.

There is, as yet, no experimental evidence to establish whether nystatin exists at the isoelectric point as a zwitterion or as an un-ionized molecule. Resolution of this question requires the examination of singly charged derivatives of the antibiotic, such as an ester and/or suitable salt. The zwitterionic nature of a closely related polyene macrolide antibiotic, amphotericin B, was lately confirmed by such techniques¹⁹⁶.

2.15 Aggregation

Molecular weight determinations with the aid of a Beckman Model E Analytical Ultracentrifuge have been performed by Kirschbaum¹¹¹ on the clear supernatant of saturated nystatin-Type A and -Type C solutions in 90% methanol/10% water at 4°, 20° and 37°C without equilibration between removal of the undissolved nystatin (by low-speed centrifugation) and the start of the MW analysis. Under these conditions, nystatin-Type A was found to exist in solution predominantly as a dimer, while nystatin-Type C is mainly a tetramer. This relationship, as established in one experiment, was maintained for solutions in equilibrium with undissolved nystatin for up to 98 hours prior to the low-speed removal of the undissolved product and subsequent MW determination on the supernatant.

From a comparison of the UV-absorption spectra of nystatin solutions in methanol/0.05% acetic acid and various aqueous buffer systems (pH 4.5, 6.8 and 9.0), Lampen *et al.*¹¹² concluded that the low extinction values typical for the aqueous media are likely to reflect that nystatin is present as micelles and is not in true solution. This inference was supported by the observation that nystatin is not dialyzable under these conditions (pH 4.5 and 6.8, 10-30 µg of nystatin per ml of 0.1% aq. dimethylsulfoxide solution), and the product could be recovered unaltered at the end of the dialysis experiment.

2.16 Polarography

A solution of nystatin in 25% aqueous ethanol, containing tetrabutylammonium hydroxide (0.15 M) as basic electrolyte, has been reported by Kramarczyk and Berg¹¹³ to be irreversibly reduced with a half-wave potential of -1.65 volts, as measured against a normal calomel electrode.

3. BIOSYNTHESIS

The structure of nystatin (see Section 1.1) is generally consistent with the biosynthetic pathway postulated for the entire class of biogenetically related macrolide antibiotics¹¹⁴, including the polyene and erythromycin sub-groups (polyketide pathway).

Isotopic tracer studies by Birch *et al.*^{49,50,115} with fermentation cultures of *Streptomyces noursei* and degradation of the resulting labelled nystatin provided evidence in support of the polyketide pathway, and also allowed for the

tentative assignment of partial structures for the nystatin molecule.

4. METHODS OF MANUFACTURE

4.1 Historical

Nystatin was first isolated by Hazen and Brown^{28,29} in 1950 from the surface growth of a liquid glucose-tryptone culture of a natural soil actinomycete (strain No. 48240) - later designated Streptomyces noursei^{33,40a,116} - originating from a farm soil specimen recovered in Fauquier County, Va.²⁹.

4.2 Microbiological Processes

While Hazen and Brown, in their original experiments leading to the discovery of nystatin, employed conventional surface culture techniques for the growth of the Streptomyces organism under static conditions²⁹, Dutcher et al.^{32,95} later succeeded in cultivating the organism by the method of deep fermentation (i.e., submerged culture, under aerobic conditions), thus providing the basis for an economical large-scale industrial production of the antibiotic.

In efforts to further improve the productivity of commercial fermentations, a large variety of yield-influencing factors - including the selection of high-productivity strains and mutants^{34,36,117-127}, modifications in media and cultural conditions¹²⁸⁻¹³⁹ most suitable for the growth of the antibiotic-producing organism, etc. - have since been explored and recorded, predominantly in the patent literature².

The original S. noursei strain (No. 48240)^{29,33,116}, several subsequently isolated mutants (generated by exposure to X-ray and UV-irradiation or after treatment with nitrogen mustard)^{118,119}, as well as specific strains of Streptomyces albulus (e.g., ATTC-12757)^{34,36} and other nystatin-producing Actinomyces organisms^{14,15,70,126,140} are known to co-produce secondary metabolites - e.g., cycloheximide (actidione)^{29,34-36,125}, antitumor antibiotic E73³⁵ - in substantial quantities under particular culture conditions.

4.3 Isolation and Purification Processes

The isolation of nystatin from culture broth¹⁴¹⁻¹⁴⁷ on industrial scale is most commonly based on extractive recovery procedures, involving (a) the admixture of an appropriate, water-miscible organic solvent to the whole fermenta-

tion broth (with or without pH adjustment), followed by (b) the removal of insoluble broth constituents via filtration, and (c) the separation of the antibiotic by either fractional precipitation or extract concentration, or suitable combinations thereof. A substantial number of reported processes^{36, 148-154} avoid the use of large solvent quantities usually required in whole broth extraction methods by first providing for the separation of a nystatin-rich mycelium cake intermediate (moist or dried) from which the antibiotic may then be extracted by any one of several suitable solvents or solvent combinations, following procedures similar to those adopted for whole broth extraction methods.

The solvents and solvent combinations most widely used in the large-scale isolation of nystatin from fermentation broths or mycelial cakes include methanol^{33,116,131,149, 151-153}, ethanol^{116,153}, n-butanol^{33,36,114,116,145,147}, n-propanol¹¹⁶, isopropanol^{116,141-143,153}, methanol/ethanol (1:1)¹¹⁶, acetone^{33,149,151}, 80% acetic acid/xylene¹⁵⁰ and pyridine¹⁵⁴.

Other, more unique recovery methods take advantage of the known ability of nystatin to form a variety of soluble complexes with inorganic salts in organic solvents - e.g., with CaCl_2 in methanol^{95,142,143}, or with NaI , NaSCN , KSCN and NH_4SCN in acetone¹⁴⁶ - which readily dissociate into the free antibiotic and the corresponding salt component on addition of water to the respective solution. Alternate isolation methods for nystatin are based on a property peculiar to its chemical nature, namely the pronounced tendency to form relatively stable aqueous emulsions with a number of water-immiscible organic solvents (alcohols, esters and ketones)^{144,145}, thus permitting a direct separation of the antibiotic from nystatin-containing broths by flotation.

A majority of the present recovery methods, however, produces relatively impure, low-potency intermediates requiring further purification, generally by procedures adapted from established broth or mycelial cake extraction techniques^{32, 117,141-146,149-152,155-160}.

5. STABILITY - DEGRADATION

Nystatin shares with many other complex polyene macrolide antibiotics a high degree of sensitivity to heat, light, oxygen, and extremes of pH, both as pharmaceutical grade bulk material in the solid state and in solution or suspension. However, very few reliable quantitative data are at

hand on the chemistry of various possible degradation processes and on the nature of the degradation product, resulting from exposure of the antibiotic to a variety of environmental conditions. Results of the few published experimental studies, listed below, often appear contradictory and are not readily interrelated, as they commonly reflect significant differences in experimental conditions (including assay methods), as well as wide variations in the origin, purity and homogeneity of the examined products (e.g., crystalline vs. amorphous product, and/or mixtures thereof).

In general terms, both the highly unsaturated nature of the molecule and the presence of a pH-sensitive lactone ring linkage undoubtedly contribute to the inherent susceptibility of nystatin to deactivation.

5.1 Dry Thermal Degradation

Among several general statements in the literature^{3, 4, 7, 39, 83, 95}, it is reported^{29, 40a} that nystatin - in the dry solid state - has been stored under refrigeration for up to 4½ years without appreciable loss of activity, but approx. 25% of its activity was lost in 6 months at 40°C under non-specified storage conditions.

5.1.1 Stability of Amorphous Product

Bashkovich and coworkers¹⁶¹ report that inactivation of amorphous nystatin, when exposed to atmospheric oxygen, is greatly enhanced by the presence of $\geq 9\%$ moisture, and suggest that loss of activity is the result of oxidative polymerization. Inactivation was also found to be increased by the presence of polyvalent metal ions (Ca^{2+} , Fe^{3+} , Cu^{2+} , Mn^{2+} , Al^{3+} , Co^{2+} and Ni^{2+}), but this effect is said to be minimized by the addition of a suitable complexing agent, such as Na-hexametaphosphate. Results of accelerated stability studies carried out by shaking nystatin powders at room temperature for 17 days in a sealed tube containing an oxygen atmosphere and exposed to UV light are reported to correlate well with the extent of deactivation after normal storage for one year at 4°C.

5.1.2 Stability of Crystalline Product

Accelerated heat stability tests conducted by Trakhtenberg et al.¹⁰⁵ with dry nystatin materials (isolated by extraction of mycelium cake with primary alcohols) showed that samples which were practically stable on storage under refrigeration nevertheless rapidly degraded at elevated

temperatures as evidenced by an activity loss of approximately 75% on storage for 3 hours at 100°C; the presence of moisture was found to enhance thermal decomposition, as was also noted by other investigators^{162,164,165}.

Kleiner and Ionova¹⁶³ examined the stability of crystalline commercial nystatin samples on heating in sealed tubes at 80° and 100°C and established first-order kinetics for the degradation under these conditions, with half-life periods of 1.33×10^3 min at 80°C, and 0.88×10^2 min at 100°C. The addition of antioxidants (e.g., thiourea and Na-metabisulfite) was found not to protect the antibiotic from thermal decomposition.

As part of an investigation to explore potential methods other than a heat-resistance test for the determination of nystatin stability, Kuzovkov et al.¹⁶⁴ studied the effect of storage under controlled humidity conditions and developed an expedient, qualitative ("express") method for stability studies. Nystatin samples were stored in open vessels over 10% H₂SO₄ in a hermetically sealed chamber at 20°C and 98% rel. humidity for a 30-day period. The authors¹⁶⁴ found that preparations otherwise shown to be unstable under normal ambient conditions lost 30-70% of their initial activity after 30 days in the high-humidity environment, while samples which were considered stable at room temperature also appeared to be stable for longer periods in the humid atmosphere. No quantitative relationship was established between the activity loss in the high-humidity environment and storage under ambient conditions. The method described appears, therefore, only useful as a qualitative test for the estimation of nystatin stability.

Lokshin et al.¹⁶⁵ provided evidence that the enhanced stability of well-dried nystatin is best preserved by storage over P₂O₅, in the absence of atmospheric oxygen. Benzoylperoxide, polyvalent transition metal ions (Fe³⁺, Co²⁺ and Cu²⁺) and high ambient humidity are reported to greatly reduce the stability and biological activity of dried products on storage at room temperature. Unidentified polymerization products, insoluble in organic solvents and in inorganic acids and bases, were shown to accumulate on prolonged storage under unprotected conditions as a consequence of aerial oxidation; mycosamine has been identified as one of the reaction products from the acid hydrolysis of the isolated polymeric constituents.

Crystalline nystatin, as opposed to the amorphous

product, and nystatin purified by treatment with Na-hexametaphosphate in aqueous isopropanol solution^{165,166} were reported to have superior stability, while being less susceptible to the deteriorating effect of humidity. The authors¹⁶⁵ suggest that aerial oxidation is the prominent cause of nystatin deactivation and also postulate that conditions of high relative humidity promote the decomposition of peroxide compounds formed during air oxidation. Among several antioxidants examined, butoxytoluene and butoxyanisole proved to be the most effective stabilizing agents.

5.1.3 Stability of Solid Dosage Forms

Thermostability tests conducted by Tebyakina *et al.*¹⁶⁷ on pharmaceutical grade samples of nystatin - as dry bulk powders and in solid dosage forms (tablets, pills) - revealed marked differences between various products after storage for up to two years at 5°C and at room temperature; while the formulated products effectively retained their original activity at both temperatures, bulk powders were subject to substantial degradation on storage, with activity losses for some samples ranging in the order of 20-30% over a 2-year storage period at 5°C. Addition of tetracycline to the dry dosage forms was reported to improve their thermal stability.

S. Boteanu and coworkers¹⁶² investigated a variety of dragee formulations under long-term storage conditions to establish a semi-quantitative relationship between excipient composition and the effects of heat exposure, relative humidity, UV- and IR-irradiation and pH on the rate of product degradation over periods of up to 720 days.

More recently, Elkouly *et al.*¹⁶⁸ compared the stability of nystatin in five different suppository bases against dry nystatin powder when stored at 5°C and 25°C. At either temperature, the dry powder was found to decompose on storage but, as expected, with a markedly lower rate at 5°C than at 25°C, in general agreement with the findings of other investigators^{105,163,167}. At both temperatures, however, it was established that biopotencies of the dry powder decreased at an appreciably faster rate during the first three months of storage (approx. 30% and 50% activity loss at 5°C and 25°C, respectively) than during the following period, consistent with early observations by Dutcher *et al.*⁸³ on lyophilized nystatin powders. The storage stability characteristics of the antibiotic in the selected suppository bases followed a similar pattern over the first 3-month period, with slightly higher initial decomposition rates at both temperatures, but near-

equal residual biopotencies after 6-month storage at either temperature (approx. 65% activity loss).

As part of this study, Elkouly and coworkers¹⁶⁸ employed, in parallel, two of the most commonly adopted quantitative procedures for the determination of potency changes during long-term storage of nystatin and its formulated products - namely a microbiological (cup-plate agar diffusion assay¹⁶⁹) and a direct spectrophotometric method (see Section 6.5) - and found very poor agreement between both procedures. In fact, the microbiological assay data provided evidence for substantial, progressive biopotency losses over the entire 6-month test period; concurrent monitoring of the UV-absorbance of nystatin at one of its three prominent absorption bands (319 nm) during the same test interval indicated effectively no absorbance changes for the storage samples at both test temperatures, thus evidently precluding the use of the direct spectrophotometric method as a reliable tool in stability studies. A similar conclusion was reached by Dutcher et al.⁸³ during early studies of the chemical and biological properties of nystatin, and has found further support in the recent findings generated by Hamilton-Miller⁹⁷ during the examination of pH and temperature effects on the stability of nystatin solutions; in addition, several other investigators^{27,96,105,170,171} have commented on the lack of a meaningful correlation between biological and spectrophotometric assays of polyene antibiotics.

5.1.4 Stability of Ointment Formulations

The stability of nystatin in twelve different ointment bases held at 37°C for various time periods (up to 75 days) was examined by Trivedi and Shah¹⁷² by the agar cup-plate method using *Saccharomyces cerevisiae* as the test organism. The degradation reaction was found to follow first-order kinetics, and half-life times are listed. Among the examined ointment bases, a composition of polyethylene glycol 400 and 4000, Span 60 and water showed maximum stability, optimum diffusion through agar and release through parchment paper.

5.2 Stability in Solution

Studies by Trakhtenberg et al.¹⁰⁵ have shown that solutions of nystatin in methanol, both under conditions of acid (0.05N HCl) and alkaline pH (0.05N NaOH), are highly unstable and lead to a near-complete loss of bioactivity within a matter of hours, without appreciable changes in the extinction attributed to the polyene chromophore.

Lokshin et al.¹⁷³ examined the kinetics of degradation for highly purified nystatin samples in anhydrous dimethylformamide solutions at several temperatures ranging from 32° to 56°C, both in the presence and absence of atmospheric oxygen. While, under these conditions, essentially no loss in biological activity was observed in the absence of aerial oxygen even after storage of the solutions at 56°C for 120 hours, rapid inactivation took place in the presence of air. Although the formation of peroxide derivatives was found to be related to the degree of deactivation, loss of bioactivity (e.g., 90% at 56°C/120 hours) showed no correlation with a concurrent decrease in UV-absorbance (e.g., only 50-60%).

The rate of autoxidation of nystatin in dimethylformamide solutions was further studied by Zhdanovich et al.¹⁷⁴ and shown to be accelerated in the presence of heavy metal ions (Fe^{3+} , Co^{2+} and, esp., Cu^{2+}), but retarded by the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT) at concentrations of 1-1.5% of the antibiotic weight.

Hamilton-Miller⁹⁷ recently investigated the effects of temperature and pH on the stability of nystatin (and amphotericin B) solutions in phosphate-citrate buffers of different pH values (range pH 3 to 8) and concluded that nystatin solutions, when held at 37°C, are optimally stable between pH 5 and 7, while rapid breakdown was observed at pH 3 and 4 (approx. 90% destruction in about 3 and 6 hours, resp.). Periodic examination by both microbiological and spectrophotometric assay methods of test solutions incubated at pH 5, 6.5 and 7 showed that the loss of biological activity proceeded at a faster rate (4 to 8 times as rapid) than did the loss of extinction characteristic of the tetraene chromophore (321, 306 and 292 nm). The author⁹⁷ suggests that the mechanism of deactivation under the selected test conditions is not determined by an epoxidation of the type established for the aerial autoxidation of other polyene macrolide antibiotics⁹². In general, loss of bioactivity followed first-order kinetics at temperatures between 37° and 100°C, except under acid conditions. Thermodynamic parameters have been calculated from the Arrhenius plots of the respective thermal stability data, and values for the apparent activation energy, entropy, enthalpy and free energy of activation characteristic for the loss of bioactivity are given.

Boudru and Bouillet¹⁷⁵ examined the stability characteristics of nystatin powders dissolved in a pH 1.6 artificial gastric medium and observed bioactivity losses at 25°C in the order of 35% after 15 min and 84% after 90 min, while an

increase of the temperature to 37°C resulted in a total loss of biological activity within 60 min. Inoculation of the same medium (pH 1.6) at 37°C with Candida albicans, however, showed a near-complete growth inhibition for the microorganism under these conditions. Identical experiments with nystatin in the form of sugar-coated tablets and powders in suspension produced similar results, leading to a complete destruction of the microorganism after 30 min and 60 min, resp., of incubation in the gastric medium.

5.3 Stability under Radiation

The use of γ -radiation to sterilize nystatin (and other polyene antibiotics and their salts) was examined by Tsyganov and Vasileva¹⁷⁶. Exposure of the antibiotic to radiation doses in the order of 10^6 rads produced satisfactory sterilization effects, but decreased the biological potency of the product by approx. 10% without, however, leading to detectable differences in the toxicity between irradiated and non-irradiated samples, neither as freshly treated specimens, nor after 1-year storage at room temperature.

5.4 Microbial Degradation

The microbial degradation of nystatin by various strains of lower pathogenic fungi has been examined¹⁷⁷, and no significant differences were found in the rate or degree of its degradation by various species of dermatophytes. However, marked differences were found in the rate of enzymatic degradation by microorganisms which were adapted and not adapted to nystatin. After a 4-hour exposure of nystatin to non-adapted strains in a suitable culture medium, approx. 70% of the antibiotic was still intact after inoculation, whereas the antibiotic was completely degraded by adapted strains during the same time period.

5.5 Stabilization

In addition to the examined stabilization methods^{161, 163, 165-167, 174} for nystatin quoted above (Sections 5.1.1, 5.1.3 and 5.2), suppository formulations of the antibiotic are reported¹⁷⁸ to be stabilized by the incorporation of mixtures of antioxidants - i.e., butylated hydroxytoluene (0.01%), butylated hydroxyanisole (0.005%) and citric acid (0.005%) - into a base consisting of lanolin/paraffin/hydrous fat (8:1:1).

Similar stabilizing effects have been attributed by Hermansky and Vondracek¹⁷⁹ to several other antioxidants, in-

cluding hydroquinone, β -naphthol, propylgallate and 2,6-di-tert-butyl-o-cresol.

6. METHODS OF ANALYSIS

6.1 Elemental Analysis

Based on the results of various structure elucidation studies of the past, several conflicting proposals for the molecular composition of nystatin have been made in the literature^{18a,31,32,46,49,50,54,83,95,105,115}. Among these, latest experimental evidence^{57,58,101} supports an elemental composition corresponding to the empirical formula $C_{47}H_{75}NO_{17}$ (MW 926.13) for the unresolved antibiotic complex; the same formula is also postulated for nystatin A₁⁵⁹, the pure main component of the nystatin complex, isolated by countercurrent distribution.

In light of the finding that nystatin is not an individual compound but rather a variable mixture of several chemically related, active constituents^{56,64,65}, present assignments for the antibiotic complex should be viewed with reserve, as illustrated by the general lack of agreement between experimental microanalytical data and the theoretical elemental composition for the proposed empirical formula (see Table VIII for a listing of elemental analyses quoted in the literature).

TABLE VIII

Elemental Analysis of Nystatin

	<u>Element</u>			
	<u>C</u>	<u>H</u>	<u>N</u>	<u>Ref.</u>
% Theory (Calculated for $C_{47}H_{75}NO_{17}$)	60.95	8.17	1.51	-
<hr/>				
% Found	58.86	8.97	1.7	32
	58.50	8.57	1.6	32
	58.42	8.18	1.66	83
	58.58	8.28	1.62	83
	58.42	8.18	1.6	95
	58.22	8.21	1.51	105
	58.21	8.26	1.75	105
	58.86	8.21	1.64	180*)

*) Squibb Res. Std. #MYNM-150-RP

6.2 Neutralization Equivalents

Nystatin has been titrated, both as a base (with perchloric acid in glacial acetic acid^{32,83,95}) and as an acid (with sodium methoxide in pyridine³² and methanol⁹⁵). The following neutralization equivalents were determined:

Neutralization Equivalents (NE)

<u>As Base</u>	<u>As acid</u>	<u>Ref.</u>
956	922	32
955,956	-	83
955	950	95
-	950	181*)

*) Squibb Res. Std. #MYNM-150-RP

6.3 Identification Tests

Nystatin may best be identified by its characteristic IR and UV absorption spectra, as well as its X-ray diffraction pattern (see Sections 2.1, 2.2 and 2.4).

The Federal Register^{75c} describes an identity test for nystatin involving the recording of the UV spectrum of nystatin in the 220-320 nm range and the determination of the absorbances at five selected absorption maxima^{182,189}.

A series of qualitative, non-specific chemical identification tests quoted in the literature² are listed below.

<u>Test</u>	<u>Response</u>	<u>Ref.</u>
Benedict	Positive	2
Carbazole	Positive	2,32,126
Molisch	Positive (Faint)	2,31,32,83,126
Schiff	Positive (Atypical)	2,32,83,126

In addition², nystatin decolorizes solutions of bromine-water¹⁸³, bromine-carbon tetrachloride^{32,126,184}, iodine-potassium iodide¹⁸³, and potassium permanganate^{32,126,184}. However, it does not give positive tests with biuret¹⁸³, Fehling^{32,83,126}, ferric chloride^{32,126,183}, Millon^{32,83,126}, ninhydrin¹⁸³, Tollens^{32,83,126}, and 2,4-dinitrophenylhydrazine⁸³ reagents.

6.4 Color Reactions

Several color reactions typical for nystatin have been reported² (see tabulation below).

<u>Reagent</u>	<u>Color</u>	<u>Ref.</u>
Hydrochloric Acid	Yellow	183
Phosphoric Acid	Pink	183
Sulfuric Acid, Conc.	Violet to Blue to Black	32,126,183,184
FeCl ₃ -K ₃ Fe(CN) ₆	Strong Blue	32,184
SbCl ₃ in Chloroform (Carr-Price)	Pink	184

Other tests suitable for the identification of nystatin involve color reactions which are common to a large number of polyene macrolides. Into this category belong the characteristic formation of a chloroform-extractable, dark yellow color constituent on heating of nystatin in sodium hydroxide solution, the transient appearance of a red-violet color with concentrated sulfuric acid, and the formation of a blue coloration on addition of concentrated hydrochloric acid or trichloroacetic acid to an alcoholic solution of nystatin¹⁸⁴.

Laubie¹⁹⁷ noted that a pink color is formed by heating an alcoholic solution of nystatin in the presence of resorcinol and concentrated hydrochloric acid (Selivanof reaction) to reflux temperature; on dilution of the mixture, the color component may be extracted into isoamyl alcohol. Although this reaction was shown to be very sensitive and may be suitable for the detection of nystatin at levels of approx. 50 µg, the method is non-specific as several other antibiotics produce similar color reactions.

A related procedure, described by the same author¹⁹⁷ and claimed to be more specific for nystatin, involves the reaction of an alcoholic nystatin solution with a mixture of concentrated hydrochloric acid and dilute aqueous ferric chloride; the intensity of the green color component formed in this reaction is reported to allow the detection of nystatin at levels identical to those quoted above. This procedure has been evaluated by Szucs¹⁹⁸ as an identity test for the determination of nystatin in the presence of a series of excipient materials commonly found in tablet formulations.

Color reactions adapted for use in the quantitative analysis of nystatin by colorimetric assay methods are covered

in Section 6.6.

6.5 Direct Spectrophotometric Analysis

The ultraviolet absorption properties of nystatin are discussed in Section 2.4.

Because of the distinct spectral fine structure of polyene macrolide antibiotics^{11,12,13,15,18a,92,93}, ultraviolet absorbance measurements are widely accepted as the most expedient tools in analytical methodology. Quantitative spectrophotometric methods for the determination of nystatin, utilizing the characteristic absorption of the conjugated tetraene chromophore with intense absorption bands near 291, 304 and 318 nm, have been employed in a variety of investigations, including the rapid differentiation of nystatin from other polyene macrolides derived from *Streptomyces* species^{11,15,18a,70}, in stability studies^{97,105,161,162,168,174} and in chemical transformations^{52,190-192}.

Although these methods were found by some authors^{96,185} to correlate acceptably with the biological activity of the antibiotic, the majority of studies^{11,27,83,96,97,105,168,170,171}, however, have established either unsatisfactory or only marginal relationships between spectrophotometric and biological assays, most likely as the result of substantial variations in the state of purity and homogeneity of the examined products, specifically with respect to differences in the ratio of active components.

The lack of an adequate agreement between both analytical methods has greatly reduced the usefulness of ultraviolet spectrophotometric procedures as tools for the assessment of product purity. Nevertheless, spectrophotometric methods are being utilized, for convenience reasons, in many process control applications^{5,96,185-187}, particularly in the measurement of nystatin concentration in fermentation broths, unpurified products and various recovery samples.

6.5.1 Fermentation Liquids and Products

The absorbance of nystatin at 304 nm has been used to determine the concentration of nystatin in fermentation broth⁵. The assay does not reflect the stability of nystatin to acid and heat, but is suitable for process control uses.

Another direct spectrophotometric assay for the determination of nystatin in fermentation broth, based on the

measurement of the difference in extinctions at 304.5 and 312 nm, is reported by Doskochilova and Gess⁹⁶. The method described is claimed to give results comparable to those obtained by the biological assay, using an actidione-resistant strain of Candida albicans (BUCAV 44) as test organism in a plate method of cultivation. Satisfactory agreement between both spectrophotometric and biological methods was reported to be maintained during the entire course of a fermentation. However, on prolonged fermentation beyond the attainment of maximum antibiotic activity, both methods begin to deviate from each other, with the biological assay indicating a sharper decline in activity of the culture fluid than reflected by the spectrophotometric method. The authors⁹⁶ explain this discrepancy with the likely decomposition of the antibiotic on extended fermentation, concurrent loss of bioactivity, but retention during decomposition of the polyene chromophore responsible for the ultraviolet absorption of nystatin.

Alternate spectrophotometric assay procedures for the determination of nystatin, developed by Sherman et al.^{186,187}, attempt to account for the presence of ultraviolet-absorbing ballast substances in fermentation liquids and unpurified intermediates which otherwise tend to affect the desired accuracy of quantitative assay methods based on extinction measurements. The proposed differential methods, applicable to both broth and isolated product samples, involve the extinction measurement of nystatin solutions (in methanol/dimethylsulfoxide mixtures) at the absorption maximum in the 302-306 nm range, plus the determination of the extinction for the minima on either side of the peak absorption, i.e., near 295 and 312 nm, respectively. Details of the quantitative procedures developed for the determination of nystatin broth and the purity of bulk product in relation to a standard sample are outlined below:

(a) Nystatin in Broth

Procedure¹⁸⁶

Measure 20 ml of well-mixed whole broth and transfer into a 6" x 1" screw-cap test tube. To deaerate the broth sample, spin for 5 min at 2000 rpm in a suitable centrifuge, and again mix the test tube contents on a Vortex Mixer for 15-30 sec.

Pipette 2 ml of the well-mixed sample into a 100-ml volumetric flask, add 75 ml of dimethyl-

sulfoxide and agitate on a rotary shaker at moderate speed for 15 min. Bring up to volume with dimethylsulfoxide, shake up by hand to mix and filter the mixture by gravity through Whatman #4 filter paper.

Pipette 2 ml of the clear filtrate into a 100-ml volumetric flask, bring up to volume with absolute methanol and mix well. Read the sample against a reagent blank (2 ml of dimethylsulfoxide, brought up to 100 ml with absolute methanol) on a suitable spectrophotometer in 1 cm silica cells. Determine the maximum absorbance for nystatin in the 302-306 nm region, and determine the absorbance at the minima on either side of this peak (in the range of 296 and 312 nm).

Calculation:

$$\frac{(A - \frac{B + C}{2}) \times D \times E}{K} = \text{Nystatin units/ml}$$

A = Absorbance at about 304 nm

B = Absorbance at about 296 nm

C = Absorbance at about 312 nm

D = Dilution factor (2500)

E = Potency of nystatin reference standard (units/mg)

K = Standardization factor determined with nystatin reference standard by the procedure outlined below.

Standardization

Weigh accurately about 5 mg of standard nystatin powder and transfer into a 500-ml volumetric flask. Add 5 ml of dimethylsulfoxide and dissolve the powder. Bring up to volume with absolute methanol and mix well. Read the standard solution against a reagent blank in 1 cm silica cells on a suitable spectrophotometer. Keep the slit width constant and maintain the same setting for sample assay. Determine the maximum absorbance of nystatin in the 302-306 nm range and the minima on each side of this peak.

Calculation:

$$\text{Standardization factor } K = \frac{(A - \frac{B + C}{2}) \times D}{\text{Weight of standard in mg}}$$

A = Absorbance at about 304 nm

B = Absorbance at about 296 nm

C = Absorbance at about 312 nm

D = Dilution factor (500)

(b) Nystatin Products

Procedure¹⁸⁷

Weigh accurately 85-105 mg of nystatin into a 100-ml volumetric flask. Add 10 ml of dimethylsulfoxide and shake to dissolve the powder. Bring up to volume with absolute methanol and mix well.

Pipette 1 ml of the clear solution into a 100-ml volumetric flask, bring up to volume with absolute methanol and mix well. Read the sample against absolute methanol as a reagent blank on a suitable spectrophotometer in 1 cm silica cells. Determine the maximum absorbance for nystatin in the 302-306 nm region, and determine the absorbance at the minima on either side of this peak (in the range of 296 and 312 nm).

Calculation:

$$\frac{(A - \frac{B + C}{2}) \times D \times E}{K \times \text{Weight of sample in mg}} = \text{Nystatin units/mg}$$

A = Absorbance at about 304 nm

B = Absorbance at about 296 nm

C = Absorbance at about 312 nm

D = Dilution factor (10,000)

E = Potency of nystatin reference standard (units/mg)

K = Standardization factor determined with nystatin reference standard by the same procedure as outlined above under (a) for nystatin in broth.

6.5.2 Pharmaceutical Preparations

Aiteanu and Medianu¹⁸⁸ examined the stability of nystatin in N,N-dimethylformamide (DMF)/ethanol mixtures and found such solutions to be stable for 24 hours, as concluded from the measurement of extinction coefficients for the absorption maxima at 291, 304, and 318 nm.

6.5.3 Other Applications

Special applications of ultraviolet spectrophotometric techniques to the examination of chemical transformations of nystatin have been reported by Bolshakova *et al.*^{52, 190}, Korchagin *et al.*¹⁹¹ and, more recently, by Udvardy *et al.*¹⁹². The latter authors examined the addition of iodine monochloride and bromine to nystatin by a combination of spectrophotometric, titrimetric and thin-layer chromatographic methods in an attempt to correlate biological activity with the tetraene content of a large number of nystatin production batches. Further details are discussed in Section 6.10.

Wayland and Weiss¹⁹³ developed a system of chemical identity tests for the specific, positive characterization of antibiotics in sensitivity disks to supplement the quantitative information obtained by microbiological assay techniques. The system is suitable for the microquantities involved in antibiotic disks, positively identifies the chemical nature of the antibiotic in an unknown disk sample and was screened for interference from other disk antibiotics. Within this scheme of chemical test procedures - involving a sequence of colorimetric, TLC and paper chromatographic tests, in combination with microbiological response and potency data - nystatin is identified by its characteristic absorption peaks at 291, 304, and 318 nm.

A general survey of spectrophotometric methods for antibiotic determination in the ultraviolet and infrared regions was published by Unterman¹⁹⁴ in 1965.

6.6 Colorimetric Analysis

Several colorimetric methods have been published for the determination of nystatin as bulk material and in pharmaceutical formulations.

The earliest methods described by Laubie¹⁹⁷ and Szucs¹⁹⁸ are semi-quantitative procedures based on the formation of distinct color components (see also Section 6.4).

Characteristic colorations are also formed upon treatment of dimethylformamide solutions of nystatin with either dilute aqueous sodium hydroxide or concentrated hydrochloric acid¹⁹⁹. The latter reaction, described by Ozsoz¹⁹⁹, producing a light-blue color on addition of 0.25 ml of concentrated HCl to a solution of 25-100 units of nystatin in 0.1 ml of DMF, has only found use as a qualitative test in the identification of nystatin, specifically in ointment formulations.

The color reaction resulting from the admixture of dilute sodium hydroxide to a DMF solution of nystatin reported by Unterman²⁰⁰, however, has been developed as a quantitative procedure suitable for the analysis of the antibiotic in tablet formulations.

Unterman²⁰¹ also found that nystatin produces a reddish-yellow color when reacted in DMF solution with $AlCl_3$, and proposed that the reaction be used as a quantitative method for the determination of the antibiotic. Ochab²⁰² later worked out optimum reaction conditions, established a linear relationship between the concentration of nystatin and the absorbance of the color component at 435 nm and - based on good agreement between colorimetric and biological assay data - adapted this method for the quantitative assay of the antibiotic in pharmaceutical dosage forms.

A different color reaction, also reported by Unterman²⁰³, involves the formation of a yellow-brown colored complex on treatment of nystatin with 6% anhydrous methanolic titanium tetrachloride solution. The absorption spectrum characteristic for this complex is different from the parent antibiotic, but retains the unique absorption maximum at 318 nm⁹⁶; the reaction has not been adapted for quantitative use. However, an apparently related color reaction described by Mazor and Papay²⁰⁴, based on the generation of a reddish-brown color complex on addition of a $TiCl_4$ solution in DMF to a nystatin solution in the same solvent, has been proposed as a method for the colorimetric determination of nystatin. The resulting complex with a molar ratio of nystatin : titanium of 1:3 exhibits strong absorbance at 450 nm and its formation obeys Lambert-Beer's law. As the colored complex no longer shows significant absorption in the ultraviolet range, the authors presume that nystatin decomposes under the conditions of the reaction and the resulting complex is, in fact, formed with one of the decomposition products.

Chang et al.²⁰⁵ have proposed a colorimetric method for the assay of nystatin, both as bulk material and in phar-

maceutical formulations, which utilizes the formation of a yellow color produced on heating DMF solutions of nystatin with aqueous sodium hydroxide. Although good agreement between colorimetric and microbiological assay results is reported, the presence of sugars is known to interfere with this method⁵. The procedure was applied to the measurement of nystatin activity in creams, ointments and capsules, and was also employed in stability studies.

A more recent colorimetric method for the determination of nystatin reported by Amer and Habib^{206,207} is based on the reaction of the alkaline hydrolysis products of nystatin with p-aminoacetophenone in the presence of concentrated hydrochloric acid.

A general colorimetric procedure proposed by Dryon¹⁸⁴ for the determination of several natural antifungal compounds (incl. nystatin, amphotericin B, and pimaricin) involves the dissolution of the polyene antibiotic in MeOH/CHCl₃ (2:1) mixtures, addition of 37% hydrochloric acid containing 20 vol.% of ethanol under cooling, formation of a blue color within ~8 min at room temperature, and photometric measurement of the extinction at 620 nm against a blank.

Korchagin *et al.*¹⁹¹ have suggested a colorimetric determination of nystatin based on the absorbance measurement of DMF-EtOH solutions following treatment with concentrated phosphoric acid for 6 min at 100°C. Photometric measurements of the stable color formed under these conditions are claimed to correlate well with direct spectrophotometric determinations and microbiological assays generated by the agar-diffusion method. The procedure has also been applied to the determination of degradation products formed on storage of methanolic nystatin solutions in the presence of acid (pH 4) and alkali (pH 9).

6.7 Chromatographic Analysis

Chromatographic methods have been widely employed in the detection and identification of nystatin, mainly as qualitative tools to differentiate the antibiotic from other known and unknown polyene antifungal agents generated by a wide variety of antibiotic-producing microorganisms, predominantly those isolated from Streptomyces species^{16,70,208,221}.

As many of the polyene antibiotics which have been isolated are known to be actually mixtures of two or more active constituents, chromatographic comparisons with previously

identified products are the most expedient means of establishing uniqueness of a newly isolated antibiotic and providing criteria for its classification.

Frequently, available chromatographic separation methods are combined with the detection of the active component on the developed chromatogram by bioautography. The application of this special detection method in paper and thin-layer chromatographic studies of antimicrobial substances as well as its general scope in the antibiotic field have been critically reviewed by Betina²¹⁴ in a recent comprehensive publication.

6.7.1 Paper Chromatography

A variety of paper chromatographic systems have been developed for nystatin, and a number of these are summarized in Tables IX and X.

The general utility of paper chromatographic methods in the differentiation of nystatin from chemically closely related polyene macrolide antibiotics produced by a large number of organisms and in their separation into individual, biologically active components from complex mixtures of similar polyenes is illustrated in several reviews^{16,70,208-210,212,213} and individual studies^{183,211,215-227}.

A simple paper chromatographic procedure for the qualitative determination of nystatin in pharmaceutical dosage forms and in admixtures with other antibiotics has been developed by Ritschel and Lercher²¹⁷.

A n-butanol/ethanol/water (5:1:4) system together with Whatman No. 1 paper has been utilized by Struyk *et al.*²¹¹ in a descending method (17-hour development) to separate nystatin from pimaricin and amphotericin A, all closely related tetraene macrolides with similar physical and biochemical characteristics.

In a related application, paper chromatography was the method of choice selected by Rao and Cullen²¹⁹ to establish the identity of one among five different active metabolic products (including antitumor antibiotic E-73) isolated from a culture broth of Streptomyces albulus.

A special paper chromatographic technique developed by Betina and Nemec^{224,225}, termed "pH-chromatography", has been applied to nystatin. This method, specifically designed

TABLE IX
Paper Chromatography Systems for Nystatin

<u>Solvent System</u> (See Table X)	<u>Paper</u>	<u>Development</u> <u>Time (hrs)</u>	<u>Method of</u> <u>Detection</u> (See Table X)	<u>R_f</u>	<u>Reference</u>
A	Whatman No. 1	15-16	1	0.25,0.32	126,215
A	Not reported	-	2	Not reported	65
A	Whatman No. 1	18	3	0.22	221,222
B	Whatman No. 1	15-16	1	0.76,0.9	126,215
C	Arches No. 302	16	4	0.58	218
D	Whatman No. 1	-	5	0.56	177
E	Whatman No. 1	15-16	1	0.73,0.63	126,215
F	Arches No. 302	18	4	0.44	218
G	Schleicher & Schüll 2043b, "hydrophobed"	15	6,7,8	Not reported	217
H	Schleicher & Schüll 2043b, "hydrophobed"	21	6,7,8	Not reported	217
I	Whatman No. 1	17	2	Not reported	211
J	Whatman No. 2	18-24	2	0.40	220,221
K	Whatman No. 1	6-7	1	0.82,0.78	126,215
L	Not reported	-	-	Not reported	219
M	Whatman No. 4	-	2	Not reported	65,67

TABLE X

Paper Chromatography Systems for Nystatin

Solvent Systems

- A n-Butanol, Water Saturated
- B n-Butanol/Acetic Acid/Water (2:1:1)
- C n-Butanol/Acetic Acid/Water (4:1:5)
- D n-Butanol/Acetic Acid/Water (4:1:1)
- E n-Butanol/Pyridine/Water (1:0.6:1)
- F n-Butanol/Pyridine/Water (2:1:2)
- G n-Butanol/Pyridine/Acetic Acid/Water (15:10:3:12)
- H n-Butanol (Water Satd.)/Ethyl Ether (Water Satd.)/Acetic Acid (5:1:1)
- I n-Butanol/Ethanol/Water (5:1:4)
- J n-Butanol/Ethanol/Water (5:1:5)
- K Acetone/Water (1:1)
- L 70% Aqueous Isopropanol
- M Methanol/Chloroform/12.5% Ammonia (1:2:1), Lower Phase

Methods of Detection

- 1 Bioautography vs. Penicillium oxalicum 99
- 2 Bioautography vs. Saccharomyces cerevisiae ATCC 9367
- 3 Bioautography vs. Saccharomyces carlsbergensis K-20
- 4 0.02N Potassium Permanganate Spray Reagent
- 5 Ultraviolet Light
- 6 9% Ferric Chloride Spray Reagent
- 7 0.25% or 0.5% p-Dimethylaminobenzaldehyde Spray Reagent
- 8 Ninhydrin-Stannous Chloride Spray Reagent

for the analysis of substances of biological origin, involves the chromatography of a selected antibiotic on a series of chromatographic paper strips buffered to pH values ranging from 2 to 10. Suitable organic solvents (e.g., n-butanol) saturated with water are used in the development of the strips by the ascending method, and the developed spots are visualized by microbiological detection. The authors^{224,225} propose this technique as a convenient means for the simultaneous determination of the ionic character of a given antibiotic and of the optimal pH values for its extraction into a suitable organic solvent (pH equal to the highest R_f value on the pH-chromatogram) and, conversely, its re-extraction from the solvent into water (pH corresponding to lowest R_f value). When applied to nystatin, the resulting pH chromatogram - generated on Whatman No. 1 paper with water-saturated n-butanol as development solvent, and covering the range of pH 2-10 - manifests the expected variations of R_f values with pH changes as anticipated for an amphoteric antibiotic, with two R_f maxima near pH 4 and pH 8, and an R_f minimum in the range pH 5-6.

In addition to the methods listed in Table X for the visualization of nystatin after development by paper chromatography either through bioautography or the use of appropriate chemical detection reagents, Litvinenko²²⁷ recorded a series of color reactions adaptable to the localization of several common antibiotics on paper chromatograms, including nystatin, and reportedly suitable for the monitoring of antibiotic concentration and purity during production.

6.7.2 Thin-Layer Chromatography

Several thin-layer chromatographic systems have been developed for the separation and identification of nystatin, primarily for use in qualitative procedures to differentiate the antibiotic from other related polyene antifungals. Some of the systems reported in the literature are summarized in Tables XI and XII.

Although the thin-layer chromatographic systems listed in Tables XI and XII have thus far only found use as qualitative methods for the separation and identification of nystatin, their generally improved resolution - in comparison to paper chromatographic techniques - has greatly enhanced the possibility to rapidly separate individual components within a complex of closely related polyene antibiotics, as recently demonstrated by Porowska and co-workers^{64,65} with the isolation of three different constituents from the nystatin complex, utilizing both thin-layer and paper chromatographic techniques

TABLE XI
Thin-Layer Chromatography Systems for Nystatin

<u>Solvent System</u> (See Table XII)	<u>Adsorbent</u>	<u>Method of Detection</u> (See Table XII)	<u>R_f</u>	<u>Reference</u>
A	Silica Gel 6060 (Eastman)	1,2	0.5	228
A	Silica Gel 6060 (Eastman) pH 2	1,2	0.5	228
A	Silica Gel 6060 (Eastman), pH 11	1,2	0.45	228
B	Silica Gel G (Merck)	3	0.66	184
C	Silica Gel G (Merck)	3	0.54	184
D	Silica Gel G (Merck), pH 8	4	0.18	229-231
E	Silica Gel G (Merck)	5	0.28	232
F	Silica Gel 6060 (Eastman)	1,2	0.22	228
G	Silica Gel GF (Analtech)	6	0.45, 0.51	233
H	Silica Gel G (Merck), pH 3	1,7,8	0.55	235
I	Silica Gel G (Merck)	5	0.53	232
I	Silica Gel G (Merck)	4	0.18	229-231
J	Silica Gel G (Merck)	1,9	0.45	221,236
K	Silica Gel G (Merck)	1,9	0.45	221,236
L	Silica Gel G (Merck)	5	0.65	232
M	Silica Gel G (Merck)	5	0.76	232
N	Silica Gel G (Merck)	5	0.63	232
O	Silica Gel 60 F-254 (Merck)	10,11	0.38, 0.40, 0.43	237
P	Silica Gel GF (Analtech)	6	0.25, 0.27, 0.32	234
Q	Kieselgur G, impregn. with 0.1M EDTA	12	0.0	238
R	Sephadex G-15, pH 6	13	0.2 (R _{rel})*	239

*Migration of nystatin relative to penicillin-G (1.0)

TABLE XII

Thin-Layer Chromatography Systems for Nystatin

<u>Solvent Systems</u>	
A	Methanol
B	Methanol/Acetone/Acetic Acid (8:1:1)
C	Methanol/Isopropanol/Acetic Acid (9:1:0.1)
D	Ethanol/Ammonia/Water (8:1:1)
E	Ethanol/Ammonia/Water/Dioxane (8:1:1:1)
F	n-Butanol/Methanol (1:1)
G	n-Butanol/Methanol/Water (5:3:2)
H	n-Butanol/Acetic Acid/Water (2:1:1)
I	n-Butanol/Acetic Acid/Water (3:1:1)
J	n-Butanol/Acetic Acid/Water (4:1:2)
K	n-Butanol/Pyridine/Water (2:1:2)
L	n-Butanol/Pyridine/Water (3:2:1)
M	n-Butanol/Pyridine/Acetic Acid/Water (15:10:3:12)
N	n-Butanol/Dioxane/Acetic Acid/Water (6:1:2:2)
O	n-Amyl Alcohol/Acetic Acid/Water (2:1:1)
P	Ethyl Acetate/Isopropanol/Water (5:5:3)
Q	Methyl Ethyl Ketone/McIlvaine Buffer, pH 4.7/Ethanol (100:6.4:22)
R	0.025M Phosphate Buffer (KH ₂ PO ₄ -NaOH, pH 6.0), 0.5M NaCl

TABLE XII (Cont'd.)

Thin-Layer Chromatography Systems for Nystatin

<u>Methods of Detection</u>	1	Ultraviolet Light ^{228,235,236}
	2	Bioautography vs. <u>Candida albicans</u> ²²⁸
	3	0.2% p-Dimethylaminobenzaldehyde Spray Reagent (in H ₂ SO ₄ , contg. trace FeCl ₃) ^{218,235}
	4	0.5% Potassium Permanganate/0.2% Bromophenol Blue Spray Reagent ²³¹
	5	5% Potassium Permanganate Spray Reagent (or H ₃ PO ₄) ²⁴²
	6	Charring with mineral acid (H ₂ SO ₄) ²³³
	7	1% p-Dimethylaminobenzaldehyde/20% SbCl ₃ Spray Reagent (in EtOH, contg. HCl) ²³⁵
	8	Ultraviolet Light (Fluorescence @ 350 nm) ²³⁵
	9	0.02N Potassium Permanganate Spray Reagent ^{221,236}
	10	Iodine/2,7-Dichlorofluorescein Spray Reagent ²³⁷
	11	Bioautography vs. <u>Candida tropicalis</u> SC 1674 ²³⁷ , or <u>Saccharomyces cerevisiae</u> SC 1600 ^{237,239,240}
	12	Chlorine/o-Toluidine Spray Reagent ²⁴³
	13	Bioautography vs. <u>Saccharomyces cerevisiae</u> ATCC 9763 ^{237,239,240}

as complementary tools.

Similar separations of the nystatin complex into its components have been achieved by Targos and Metzger²³³ and Kocy and Cole²³⁷.

Nussbaumer²³⁶ proposed a TLC procedure to establish the degree of nystatin degradation in the formulated drug by monitoring the appearance of a primary oxidation product with an R_f value of 0.73-0.75, compared to an R_f value of 0.45 for the intact antibiotic.

A unique application of thin-layer chromatography has been reported by Zuidweg et al.²³⁹ with the use of Sephadex G-15 as adsorbent medium. Instead of organic solvent mixtures, this medium utilizes an aqueous buffer solution as the developing agent, thus avoiding the possible formation of false inhibition zones during bioautographic development due to incomplete removal of solvent. By combining Sephadex TLC with bioautography (against *S. cerevisiae* ATCC 9763 for nystatin), the authors²³⁹ accomplished the often problematic separation and qualitative analysis of antibiotics mixtures, including nystatin, amphotericin B, and various penicillins and tetracyclines.

Combinations of paper and thin-layer chromatographic methods have been applied by Zhdanovich et al.²²⁶ to the separation and identification of decomposition products of nystatin arising from the partial and total oxidation of the antibiotic with $KMnO_4$ in acidic media. Some of the identified products - including succinic, formic, malic and lactic acid - are also claimed to be formed as secondary decomposition products during the natural degradation of nystatin on storage.

In an effort to overcome the mechanical problems associated with the need to provide a proper surface contact between the inoculated agar layer and the rigid, glass-backed TLC plate in bioautographic detection methods²¹⁴ for antimicrobial substances, Meyers and Smith²⁴⁰ introduced the use of spread-layer chromatograms and developed a now commonly adopted transfer technique which consists of inserting a sheet of filter paper between the TLC plate and agar surface. The resulting sandwich is incubated overnight at 37°C with the chromatographic plate and filter paper contacting the agar layer. This method produces sharp, well defined antibiotic zones of inhibition, with sensitivities comparable to those realized with paper chromatograms. Basic alumina, neutral alumina, and silica gel H were found to be suitable adsorbent media for this technique. In the bioautography of nystatin,

S. cerevisiae served as a useful indicator organism. Several later modifications of this detection method are reported²²¹.

For the determination of the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT) in admixture with commercial nystatin in pharmaceutical bulk materials, a TLC procedure has been proposed by Korchagin et al.²⁴¹.

6.7.3 Gas-Liquid Chromatography

As part of a comprehensive study to establish a general analytical screening scheme for a wide range of materials encountered in forensic toxicology (common poisons, drugs, and human metabolites), Finkle et al.²⁴⁴ developed a simple GLC system, utilizing four different columns and three liquid phases, to detect any one of almost 600 different substances, including nystatin, to a sensitivity limit of 2 µg/ml in blood, urine and tissue specimens.

During the examination of several polyene antifungal antibiotics by pyrolysis-gas chromatography, Burrows and Calam²⁴⁵ have shown that nystatin and amphotericin B can be distinguished from each other and from three other polyene macrolides (candicidin, levorin and trichomycin) by the gas chromatograms of their pyrolysis products.

6.7.4 High Performance Liquid Chromatography

Lately, high performance liquid chromatography has been employed in several instances to separate and characterize the individual components of macrolide antibiotic complexes with similar chemical structure²⁴⁶⁻²⁴⁸.

In efforts specifically aimed at the development of a rapid separation method applicable to all chromophore classes of the polyene macrolide antifungal antibiotics, Mechlinski and Schaffner^{247,248} recently applied a high-speed liquid chromatography (HSLC) technique to the analysis of several prominent polyene antibiotics, including nystatin. In brief, the reported procedure involves the use of a non-commercial liquid chromatograph composed of a Milton Roy high-pressure reciprocating pump with pulse dampener connected to a septum injector, followed by a chromatographic column, a 350 nm UV monitor and waste reservoir.

The separation of the nystatin complex was achieved in a reverse-phase mode with a mixture of water/methanol/THF (420:90:60 or 420:90:50) as the mobile phase, resulting in the

isolation of three distinct polyene components, two of which - including the main component - were identified as tetraenes, while the third constituent proved to be a heptaene macrolide by spectrophotometric examination. The entire analysis was completed within approx. 15 min, with a retention time for the main component of approx. 4-5 min with both mobile phase solvent mixtures. Possible adaptation of the procedure for use in the quantitative analysis of the individual components is indicated and may require an adjustment in detector response, possibly by increasing the sensitivity of the instrumentation through the use of a continuously variable wavelength UV detector which would allow each chromophore to be monitored at its respective absorption maximum.

6.8 Electrophoretic Analysis

Paris and Theallet²¹⁸ separated a number of antibiotics, including nystatin, by high-voltage paper electrophoresis on Arches 302 paper at a potential gradient of 15.3 volts/cm over a 2-hour period. With 5% aqueous formic acid solution (pH 2) as electrolyte, nystatin showed a displacement toward the cathode of 13 mm in 2 hours and, over the same timeperiod, a migration of 17 mm toward the anode in an alkaline Veronal buffer solution (pH 8.6). In the separation of complex antibiotics mixtures, the use of paper electrophoresis at different pH ranges is suggested as a supplemental technique to ordinary chromatographic methods.

Electrophoretic mobilities of nystatin, amphotericin A, amphotericin B and several other antibiotics in various different electrolyte systems (salt solutions and solvents) are also reported²⁴⁹.

6.9 Polarographic Analysis

The use of polarography in the determination of antibiotics has been discussed in a recent review by Unterman and Weissbuch²⁵⁰.

As outlined in Section 2.16, the polarographic behaviour of nystatin has been examined¹¹³.

Icha and Strosova²⁵¹ have reported the determination of nystatin content in the fermentation medium, mycelium and bulk product by oscillopolarographic evaluation of its degradation products resulting from alkali treatment.

6.10 Titrimetric Analysis

From a series of potentiometric titrations of nystatin with either glacial acetic acid or mixtures of glacial acetic acid and benzene, dioxane or chloroform as solvent media, and perchloric acid in acetic acid or dioxane as titrants, Mazor and Papay²⁵² evolved an optimum set of conditions for the titration of nystatin in non-aqueous media. The best results for the determination of the antibiotic by both potentiometric and visual endpoint titrations have been obtained with a solution of 5-50 mg of nystatin in 15 ml of a 1:14 (v/v) mixture of glacial acetic acid/dioxane and titration with standard 0.01N perchloric acid in dioxane, using either a glass-calomel electrode combination in a potentiometric procedure, or a visual endpoint determination with methyl violet as indicator. Each ml of 0.01N HClO_4 is equivalent to 9.52 mg of nystatin.

In applying this procedure to the molecular weight determination of nystatin, the authors²⁵² obtained an equivalent weight of 952 for a purified sample of nystatin (see Section 6.2). It is also stressed that the results of potentiometric titrations of nystatin will not provide any measure for the biological activity of a given sample.

Attempts at utilizing the addition of bromine or iodine monochloride as the basis for a direct titrimetric determination of nystatin in glacial acetic acid have been reported by Udvardy *et al.*¹⁹²; however, in either case it was found that halogen addition to the olefinic linkages of nystatin fell short of the theoretically calculated values for six double bonds over a wide range of experimental conditions. Nevertheless, at 105°C and a reaction period of 2 min., iodine monochloride uptake was shown to be equivalent to the saturation of four double bonds. A quantitative version of the latter reaction - involving the dissolution of nystatin in a glacial acetic acid/sulfuric acid mixture, reaction with an excess quantity of a 0.1N iodine monochloride solution, addition of excess potassium iodide solution after the reaction and, finally, back-titration with 0.1N sodium thiosulfate solution - was adopted by the investigators as a means to estimate the tetraene content of a large number of nystatin batches in an effort to correlate the results of chemical assays with biological activity determinations.

6.11 Microbiological Methods

Agar diffusion microbiological assays are in general

use by regulatory agencies^{42,253,254} for the determination of nystatin in pharmaceutical products. Turbidimetric, tube dilution and respiration inhibition procedures, as well as automated methods, are discussed in respective reviews^{5,169,170,255,256}. In addition to these conventional antibiotic assay procedures, nystatin activity assays based on its mode of action (membrane disruption, followed by cytoplasmic leakage) have been proposed. They include the measurement of specific conductance changes resulting from the efflux of ionic intracellular constituents²⁵⁹, the analysis of released potassium ions²⁶⁰ and of yeast cell constituents, specifically ninhydrin-positive amine products²⁶¹; the latter method is an automated procedure.

Nystatin in animal feeds is measured by an agar diffusion method following extraction with methanol^{257,258}. Determination of nystatin in blood, other body fluids, animal tissues and pharmaceutical dosage forms has been described and reviewed^{169,256}. Sensitivity of the agar diffusion method is approx. 3 units per ml of blood serum, and that of the micro-scale turbidimetric method is approx. 1 unit per ml.

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The above references attempt to cover the literature through 1972 (Chemical Abstracts, Volume 76). In addition to several more recent publications also included, the following additional papers related to analytical aspects of nystatin have come to the author's attention during the preparation of this profile:

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Application of orthogonal functions to determination of nystatin in the presence of its degradation products
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Ionization of acid-base groups of polyenic antibiotics in aqueous solutions
Antibiotiki 20, 678 (1975).
265. V.A. Weinstein et al.
Studies on association of nystatin and amphotericin B in non-aqueous solvent systems
Antibiotiki 20, 688 (1975)
266. E. Jereczek et al.
Use of tris (dipivaloylmethane) europium in NMR studies of some structural elements of antibiotics of the polyene macrolide group
Inst. Nucl. Phys., Cracow, Rep. 1973, No. 819/(PL) (Pt. 2), 232.

8. ACKNOWLEDGMENT

The author expresses his appreciation to Dr. T.B. Platt for his contribution of the section on microbiological assay methods; to Dr. N.S. Semenuk and his associates of the Science Information Department of the Squibb Institute for Medical Research for their assistance in the literature search; to Ms. E. Fralick for a thorough review of the manuscript; and to Ms. F. Kaiser for her expert secretarial support and for her patience in the preparation and correction of this monograph.

PROPARACAINE HYDROCHLORIDE

Daisy B. Whigan

TABLE OF CONTENTS

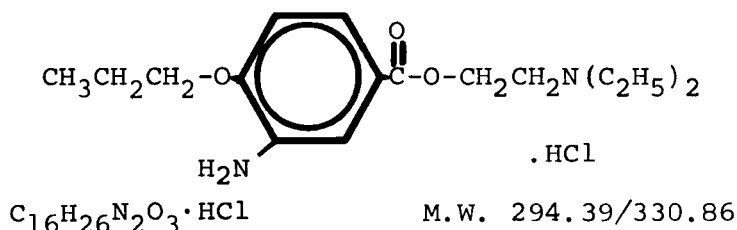
1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor
2. Physical Properties
 - 2.1 Spectra
 - 2.11 Infrared Spectra
 - 2.12 Nuclear Magnetic Resonance Spectra
 - 2.13 Ultraviolet Spectra
 - 2.14 Mass Spectra
 - 2.15 Fluorescence Spectra
 - 2.2 Crystal Properties
 - 2.21 Crystallinity
 - 2.22 Polymorphism
 - 2.23 Differential Thermal Analysis
 - 2.24 Thermal Gravimetric Analysis
 - 2.25 Differential Scanning Calorimetry
 - 2.26 X-Ray Powder Diffraction
 - 2.27 Melting Range
 - 2.3 Solution Data
 - 2.31 Solubility
 - 2.32 pKa
 - 2.33 Phase Solubility Analysis
3. Synthesis
4. Stability-Degradation
5. Analysis of Intermediate Compound and Hydrolysis Products
6. Methods of Analysis
 - 6.1 Identification Tests
 - 6.2 Elemental Analysis
 - 6.3 Spectrophotometric Analysis
 - 6.31 Ultraviolet Spectrophotometric Analysis
 - 6.32 Fluorescence Spectrophotometric Analysis
 - 6.4 Titrimetric Procedures
 - 6.41 Nonaqueous Titration
 - 6.42 Titration with Sodium Nitrite
 - 6.43 Spectrophotometric Titration with Nitrous Acid
 - 6.5 Colorimetric Methods
 - 6.51 With Bratton-Marshall Reagent

- 6.52 With Sodium 1,2-Naphthoquinone-4-sulfonate
- 6.6 Chromatographic Procedures
 - 6.61 Paper Chromatography
 - 6.62 Thin-layer Chromatography
- 7. Analysis of Hydrolysis Products in Body Fluids and Tissues
- 8. Serum Protein Binding
- 9. Drug Metabolism
- 10. References

1. Description

1.1 Name, Formula, Molecular Weight

Proparacaine hydrochloride is 2-(diethylamino)ethyl 3-amino-4-propoxybenzoate monohydrochloride. Chemical Abstracts listings are under the heading benzoic acid, 3-amino-4-propoxy-2-(diethylamino)ethyl ester, monohydrochloride. The Chemical Abstracts Registry Number is 5875-06-9. It is also known as proxymetacaine hydrochloride. Common trade names are Ophthaine, Alcaine, and Ophthetic.



1.2 Appearance, Color, Odor

Proparacaine hydrochloride is a white or faint buff crystalline, odorless powder.

2. Physical Properties

2.1 Spectra

2.11 Infrared Spectra

The infrared spectrum of proparacaine hydrochloride compressed in a potassium bromide pellet is shown in Figure 1. The spectrum was obtained on a Perkin-Elmer Model 621 grating infrared spectrophotometer. The following assignments have been made for structurally significant bands²:

<u>Wavelength, cm⁻¹</u>	<u>Assignment</u>
3420, 3280	NH ₂ stretch
2700, 2640	HCl
1700	Ester C=O
1610, 1585, 1510	Aromatic C=C
1295, 1200	=C-O(ester and aromatic ether)

Monguzzi²³ obtained the infrared spectrum of proparacaine hydrochloride from a mineral oil dispersion on a Perkin-Elmer spectrophotometer Model 157. The following spectral assignments were made:

<u>Wavelength, cm⁻¹</u>	<u>Assignment</u>
3470	-NH ₂ group
3300	-NH ₂ group
2620	N ⁺ H of trisubstituted amine
2500	N ⁺ H of trisubstituted amine
1715	C=O Ester
1630	Phenyl ring
1600	Phenyl ring
870	CH aromatic

The discrepancies in the spectral wavelengths of the two interpretations could be attributed to calibration differences of the different instruments used².

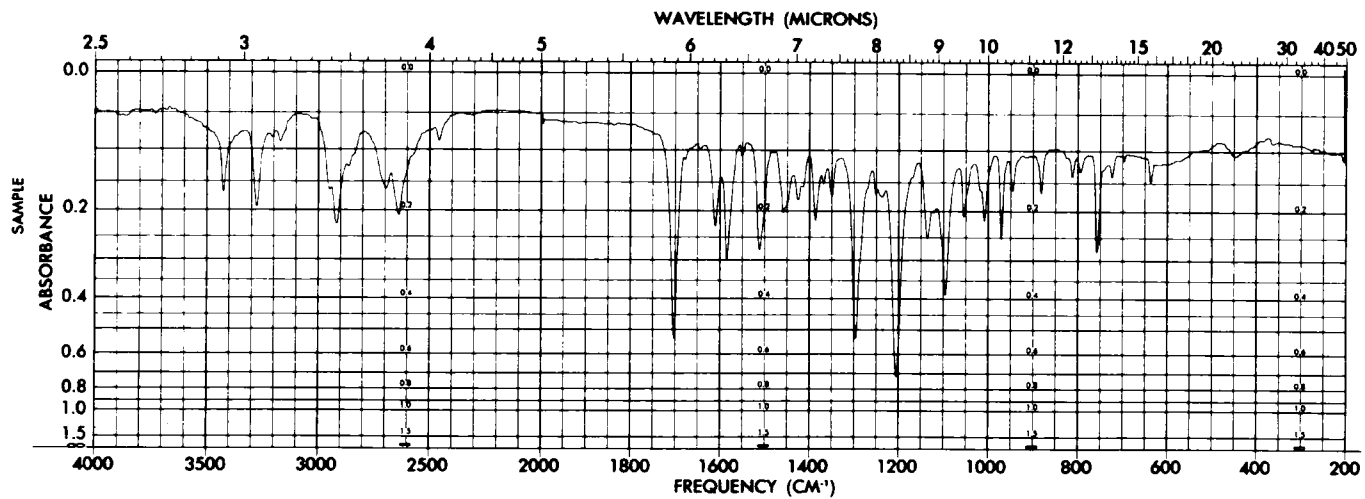
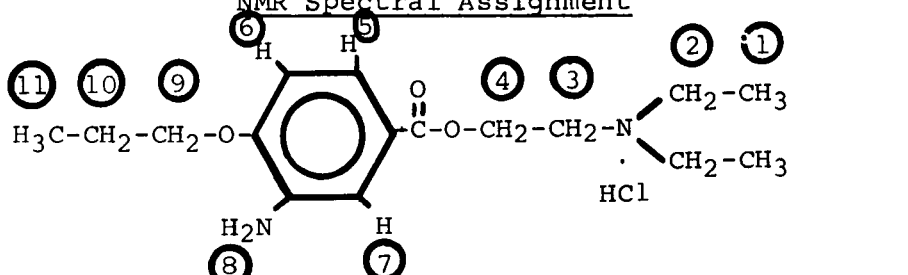


Figure 1. Infrared Spectrum of Proparacaine Hydrochloride in a KBr Pellet.

2.12 Nuclear Magnetic Resonance Spectra

Figure 2 shows the nuclear magnetic resonance spectrum of proparacaine hydrochloride in deuterated dimethylsulfoxide. The spectrum was obtained on a Perkin-Elmer R12B NMR spectrometer using tetramethylsilane as an internal reference. Spectral assignments³ are recorded in Table 1.

Table 1 NMR Spectral Assignment		
		
Proton Position	Chemical Shift δ , ppm (No. of Peaks)*	Coupling Constant J (Hz)
1	1.27 (t)	7.0
2	3.17 (q)	7.0
3	3.44 (t)	6.0
4	4.61 (t)	6.0
5	7.32 (q)	9.0
6	7.40 (d)	9.0
7	6.90 (d)	1.0
8	5.00 (b)	---
9	4.00 (t)	6.0
10	1.75 (m)	---
11	1.00 (t)	6.5
N ⁺ H	11.35 (b)	---

* d = doublet, t = triplet, q = quartet,
m = multiplet, b = broad

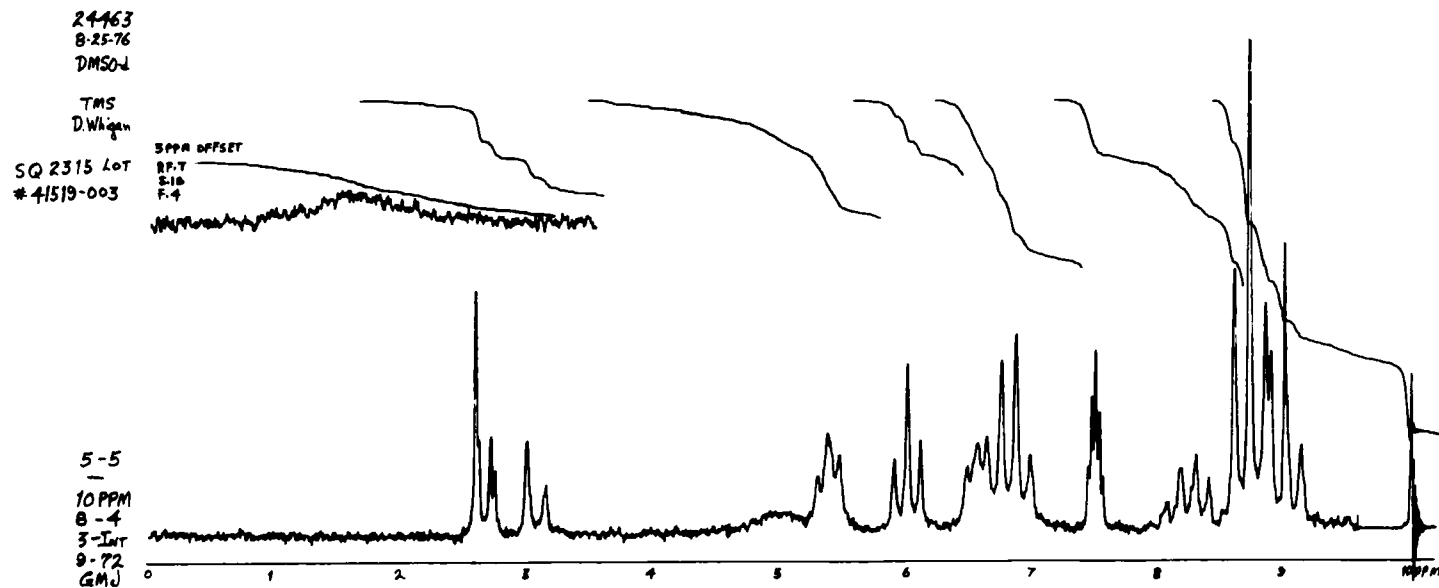


Figure 2. NMR Spectrum of Proparacaine Hydrochloride in Deuterated Dimethylsulfoxide.

2.13 Ultraviolet Spectra

The ultraviolet spectrum of proparacaine hydrochloride in methanol, ca. 12 $\mu\text{g/ml}$, is shown in Figure 3 (Instrument: Cary 15). Hefferen and co-workers⁸ attributed the following chemical structures responsible for the ultraviolet absorption of substituted benzoic acid esters:

Chemical Structure	Approximate Wavelength, nm
Carbonyl directly attached to aromatic ring	225
Amino conjugated with carbonyl	300
Ethers conjugated with carbonyl	270

The ultraviolet maxima observed for proparacaine hydrochloride agree very well with the above assignments. All three peaks are also observed when ethyl alcohol⁶, water^{5,7}, and aqueous base⁴ are used as solvent instead of methanol.

Figure 4 shows that the ultraviolet absorption of proparacaine is dependent on pH. The effect of the pH of the solution on the ultraviolet absorption of proparacaine hydrochloride was extensively studied by Hefferen⁸. At acidic pH, the aromatic amine forms a positively charged ammonium ion thus nullifying the participation of the amino group in resonance with the aromatic ring. The pH profile of the spectra presented by Hefferen showed an isobestic point at 243 nm.

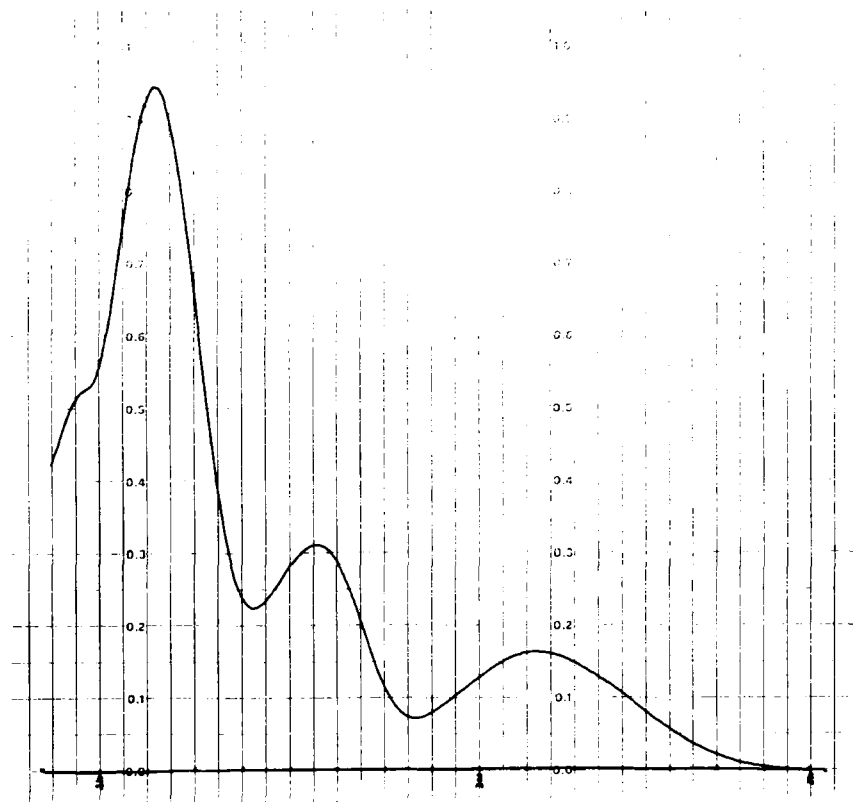


Figure 3. Ultraviolet Spectrum of Proparacaine Hydrochloride
Solvent:Methanol - Instrument:Cary 15

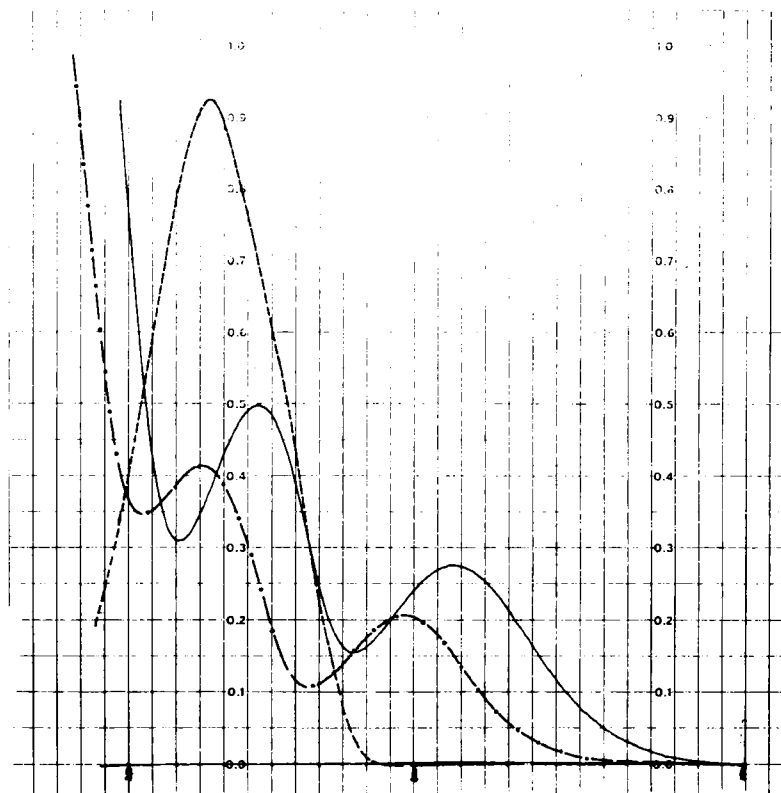
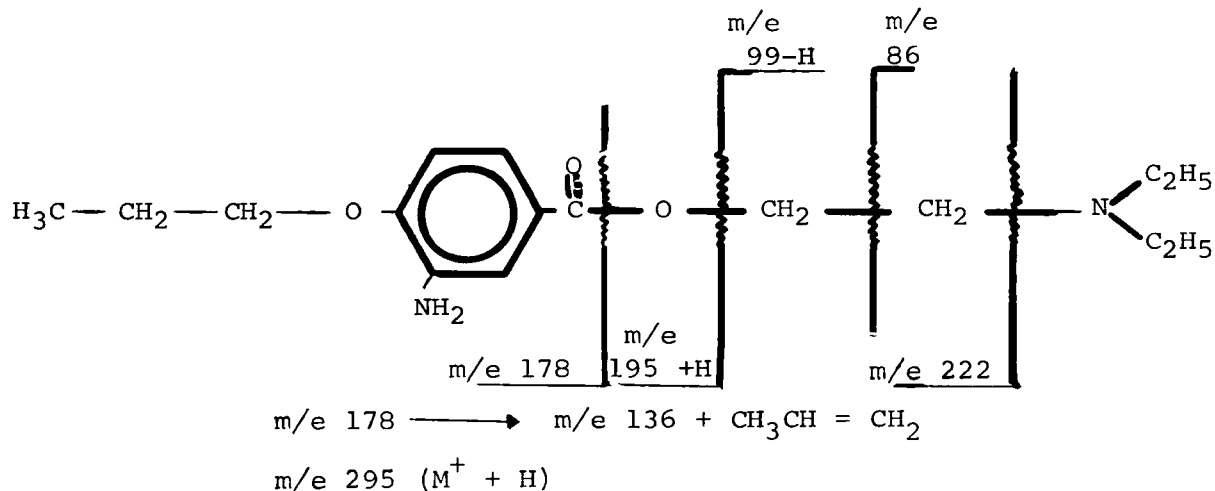


Figure 4. Ultraviolet Spectra of Proparacaine Hydrochloride in
 a) — pH 7.0 phosphate buffer b) ---- 0.1N Hydrochloric Acid
 c) -.-.- 0.1N Sodium Hydroxide Instrument: Cary 15

2.14 Mass Spectra

The low resolution mass spectrum of proparacaine hydrochloride, Squibb Standard Lot 41519-003, is shown in Figure 5. This was obtained on an Associated Electrical Industries Model MS-902 Mass Spectrometer equipped with a frequency-modulated analog tape recorder.

The parent ion, M^+ , of the compound at m/e 294 is weak. The major ion at m/e 86 is due to the cleavage of the bond beta to the tertiary amine nitrogen. This cleavage is anticipated in the fragmentation of amines. Mass spectral assignments of prominent ions are given by the fragmentation pattern below⁴⁸.



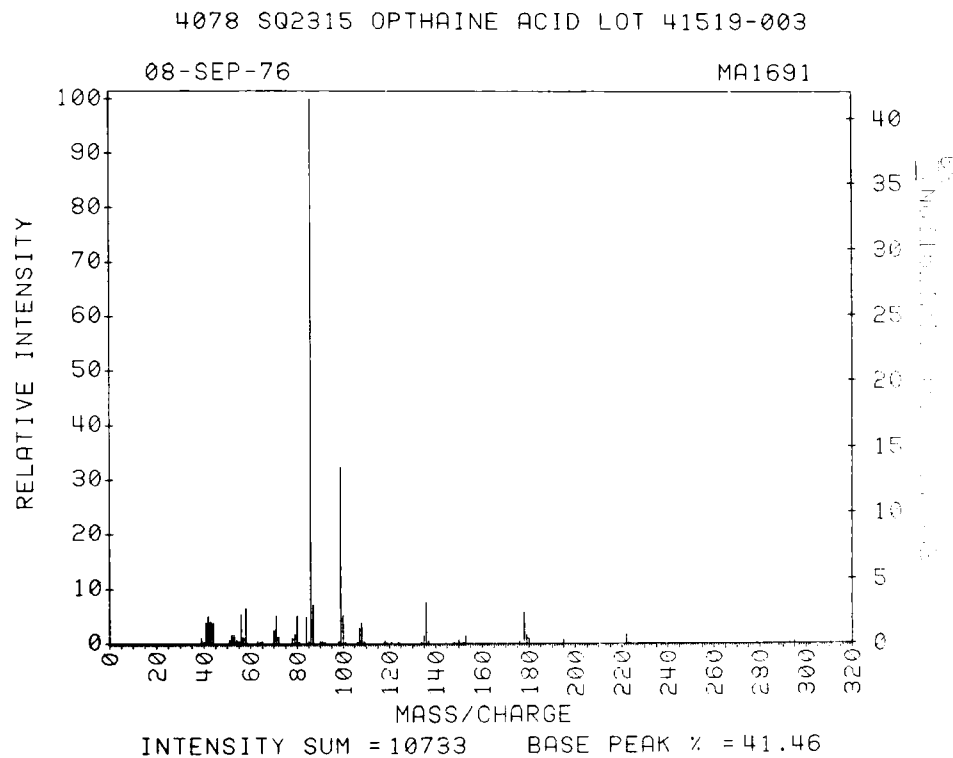


Figure 5. Low-Resolution Mass Spectrum of Proparacaine Hydrochloride

2.15 Fluorescence Spectra

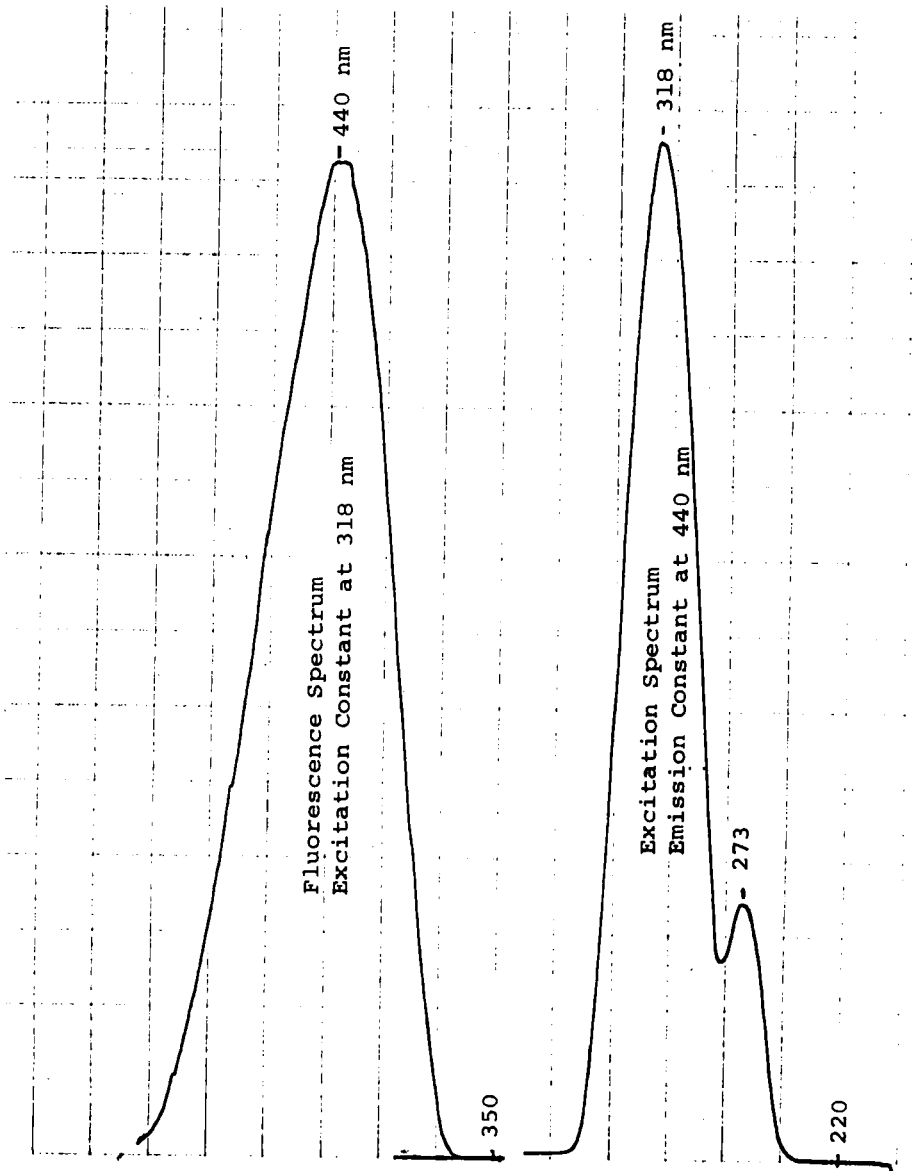
Proparacaine hydrochloride exhibits native fluorescence⁴. Its excitation and fluorescence spectra in methanol as recorded on a Perkin-Elmer Fluorescence Spectrophotometer Model 204 are reproduced in Figure 6. The fluorescence of proparacaine hydrochloride varies with pH. It is most intense in 0.1N sodium hydroxide where a concentration of 0.05 μg per ml had an intensity that was five times that of the blank. In 0.1N sulfuric acid, the fluorescence is quenched. Fluorescence characteristics of proparacaine hydrochloride in a limited list of solvents are presented in Table 2.

Table 2
Fluorescence Characteristics of
Proparacaine Hydrochloride

<u>Solvent</u>	<u>Excitation</u> <u>Maximum, nm</u>	<u>Fluorescence</u> <u>Maximum, nm</u>
Water	316	460
Sodium Hydroxide, 0.1N	300	396
Phosphate Buffer, pH 7.0	316	454
Methanol	318	440

In 0.1N sodium hydroxide, there is a linear relationship between the fluorescence intensity and the concentration of proparacaine hydrochloride up to 5 μg per ml.

Figure 6. Excitation and Fluorescence Spectra of
Proparacaine Hydrochloride
Instrument: Perkin-Elmer Fluorescence
Spectrophotometer Model 204
Solvent: Methanol



2.2 Crystal Properties

2.21 Crystallinity

Proparacaine hydrochloride forms small rosettes or bunches of needles with platinic bromide⁹. It also forms rosettes of long thick needles with 5-nitrobarbituric acid⁴⁴. Photomicrographs of crystals formed with chloroplatinic acid, picrolonic acid, and potassium permanganate were taken by Rich and Chatten¹⁰.

2.22 Polymorphism

No polymorphism has been reported for proparacaine hydrochloride. However, Koehler and Feldmann¹¹ suggested the possibility of polymorphism in the solid tetraphenylborate derivative.

2.23 Differential Thermal Analysis (DTA)

Jacobson¹² conducted the differential thermal analysis of proparacaine hydrochloride on a DuPont 900 Thermo-analyzer with a temperature rise of 15° per minute. The thermogram of proparacaine hydrochloride (Squibb House Standard Lot 41519-003) showed a sharp endotherm at 181°C which corresponds to the melt of the drug (See Section 2.27 for Melting Range).

2.24 Thermal Gravimetric Analysis (TGA)

Thermal gravimetric analysis of proparacaine hydrochloride was conducted on a DuPont Thermogravimetric Analyzer Model 900. Working with proparacaine hydrochloride, Squibb Standard Lot 41519-003, Jacobson¹² found no weight loss before 150°C. The compound was heated at a rate of 15° per minute under a nitrogen sweep.

2.25 Differential Scanning Calorimetry (DSC)

Valenti¹³ determined the purity of proparacaine hydrochloride by DSC. A scanning rate of 0.625 deg/min and a sensitivity of 2 millical/sec were used. Using a Perkin-Elmer DSC Model 1B, the purity of proparacaine hydrochloride lot 46016-064

was calculated to be 99.94 mol percent.

2.26 X-Ray Powder Diffraction

The x-ray powder diffraction pattern of proparacaine hydrochloride was obtained by Ochs¹⁴ on a Phillips X-Ray Powder Diffractometer, Type 120-101-11, at a voltage of 35 kv and a current of 10 mA. The sample was irradiated by a copper source at 1.54 Å. Data derived from the spectrum (Figure 7) of proparacaine hydrochloride, Squibb Standard Lot 41519-003, are listed in Table 3.

Table 3
X-Ray Powder Diffraction Pattern of
Proparacaine Hydrochloride

Instrument: Phillips X-Ray Powder Diffractometer

$I(2\theta)^*$	$d^{\circ}(\text{Å})^{**}$	I/I_o^{***}
7.04	12.56	0.663
10.02	8.83	0.460
11.29	7.84	0.176
12.74	6.95	0.197
13.59	6.52	1.000
15.63	5.67	0.212
16.22	5.46	0.140
17.33	5.12	0.178
17.84	4.97	0.357
19.62	4.52	0.518
21.32	4.17	0.483
23.36	3.81	0.179
24.47	3.64	0.299
25.15	3.54	0.122
26.42	3.37	0.360
27.02	3.30	0.300
27.36	3.26	0.497
29.31	3.05	0.261
33.31	2.69	0.153
33.82	2.65	0.226
35.77	2.51	0.115

*Twice the angle of incidence or reflection

** $d(\text{interplanar distance}) = \frac{n\lambda}{2 \sin \theta}$

$$\lambda = 1.539 \text{ Å}$$

***Relative intensity based on highest intensity of 1.000.

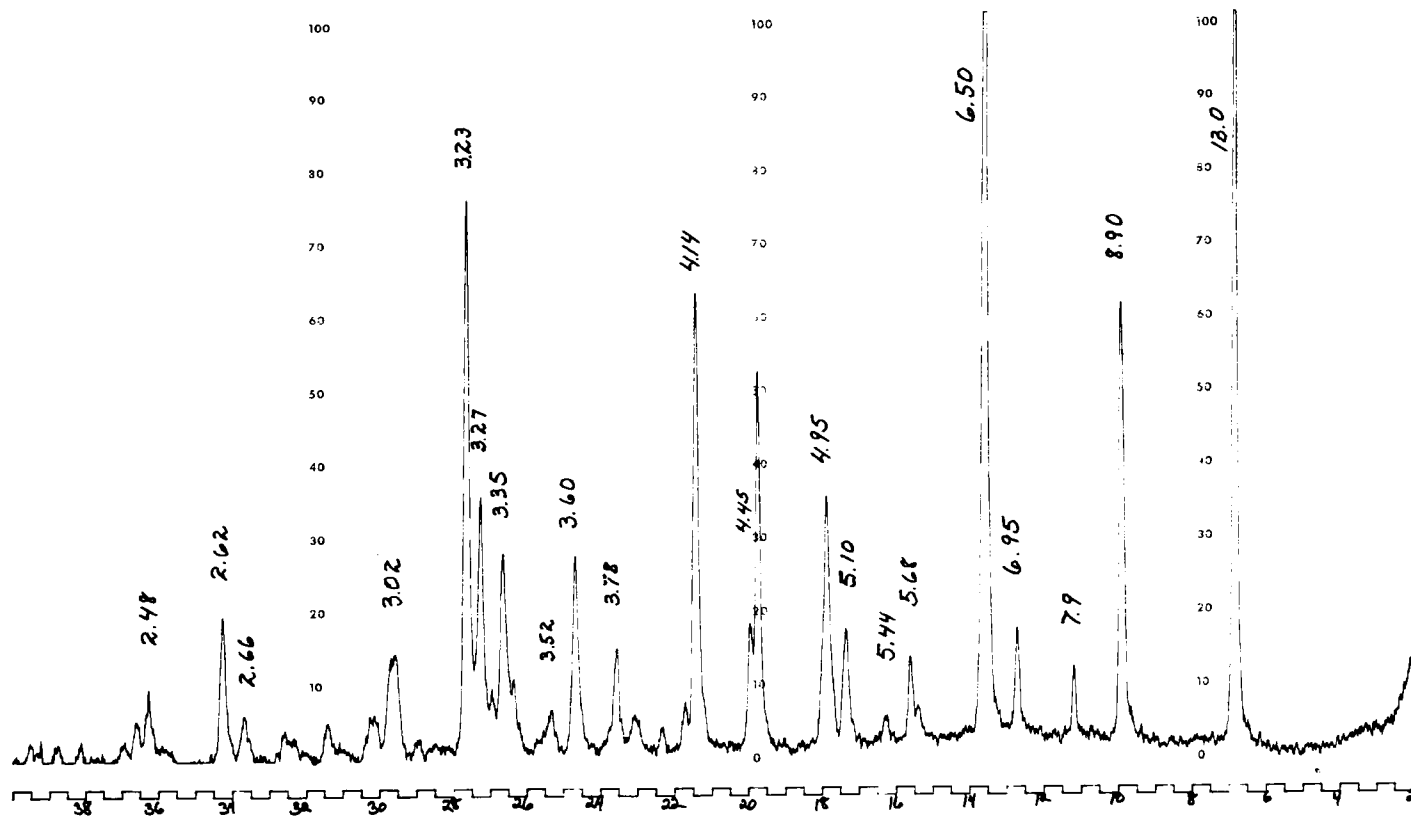


Figure 7. X-Ray Powder Diffraction Pattern of Proparacaine Hydrochloride

2.27 Melting Range

The melting range for U.S.P. proparacaine hydrochloride is specified as 178° to 185°C.¹ Clinton, *et.al.*¹⁵ reported a melting range of 182-183.3°C. Squibb Standard proparacaine hydrochloride Lot 41519-003 gave a melting range of 182° to 184°C. Monguzzi and co-workers²³ reported a melting range of 180° to 182°C.

2.3 Solution Data

2.31 Solubility

Approximate Solubility of Proparacaine Hydrochloride at Room Temperature¹⁷

<u>Solvent</u>	<u>Solubility (mg/ml)</u>
Water	> 50
Dimethylsulfoxide	50
Chloroform	30
Ethanol	7
Benzene	< 0.1
Hexane	< 0.1
Ethyl Acetate	< 0.1
Ether	< 0.1

2.32 pKa

Hefferen⁸ determined the apparent dissociation constant of the aromatic amino group:



Using a spectrophotometric method described by Flexser, Hammett, and Dingwall¹⁸, the apparent pK'a is 3.22 ($K_a = 6.03 \times 10^{-4}$)

2.33 Phase Solubility Analysis

The purity of proparacaine hydrochloride has been determined by phase solubility analysis⁶. The analysis is carried out by equilibration in absolute ethanol at 23°C for 24 hours. Proparacaine hydrochloride Lot No. BR-1 assayed 99.8% pure by phase solubility analysis.

3. Synthesis

Proparacaine hydrochloride has been synthesized¹⁹ by the sequence of reactions shown in Figure 8. The four step synthesis starts with p-hydroxybenzoic acid. This is etherified with n-propylbromide in the presence of potassium hydroxide. The resulting compound is nitrated to give 3-nitro-4-propoxybenzoic acid (II). The acid chloride is formed with thionyl chloride and reacted with β -diethylaminoethanol to yield 2-(diethylamino)ethyl 3-nitro-4-propoxybenzoate (III). This intermediate is reduced with hydrogen catalytically, to produce proparacaine hydrochloride(IV).

Clinton and co-workers¹⁵ synthesized 3-nitro-4-propoxybenzoic acid (II) by alkylation of 4-hydroxy-3-nitrobenzoic acid with propyl p-toluenesulfonate in xylene solution. The free acid is produced by subsequent alkaline saponification of the ester.

Following the Williamson reaction, Monguzzi and co-workers²³ obtained II directly from 4-chloro-3-nitrobenzoic acid by reacting it with sodium n-propoxide in dimethylsulfoxide solution.

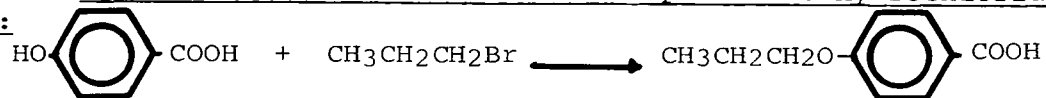
The nitro group in the intermediate III has also been successfully converted to the corresponding amino group by

- a) iron-hydrochloric acid reduction^{15,20}
- b) catalytic hydrogenation with palladium/charcoal catalyst^{22,23}
- c) catalytic reduction with Raney nickel²⁴
- d) catalytic reduction with platinum oxide¹⁵

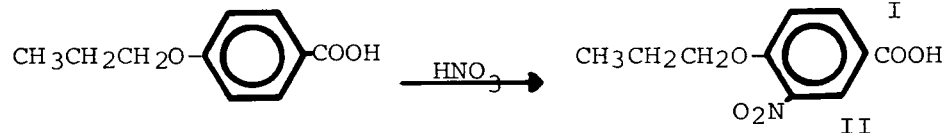
Proparacaine hydrochloride has been recrystallized from absolute ethanol²⁰, from methanol¹⁹, and from absolute alcohol-ethyl acetate¹⁵.

Figure 8. Synthetic Reaction Scheme for Proparacaine Hydrochloride

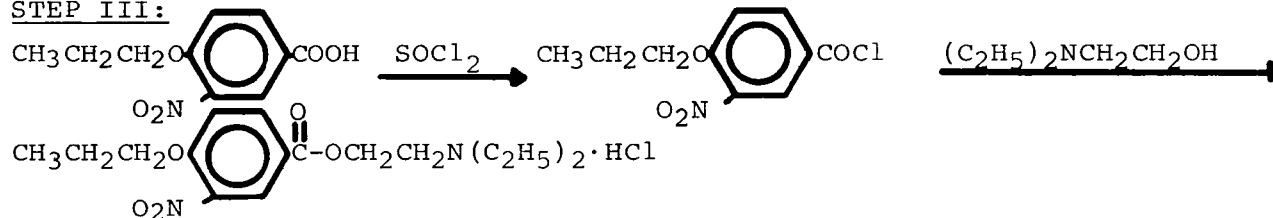
STEP I:



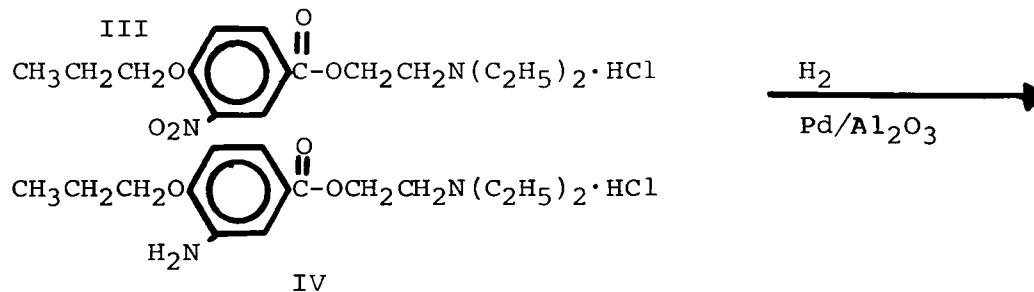
STEP II:



STEP III:



STEP IV:



Proparacaine hydrochloride is chemically stable as a solid at room temperature for at least two years²⁵. The white crystalline powder discolors on heating and exposure to air⁹. Liquid formulations of proparacaine hydrochloride are stable up to at least two years¹⁶ in the absence of air. However, solutions will discolor in the presence of air²¹.

Proparacaine hydrochloride undergoes hydrolysis when boiled in 2N hydrochloric acid for 60 minutes²⁶ (Figure 9).

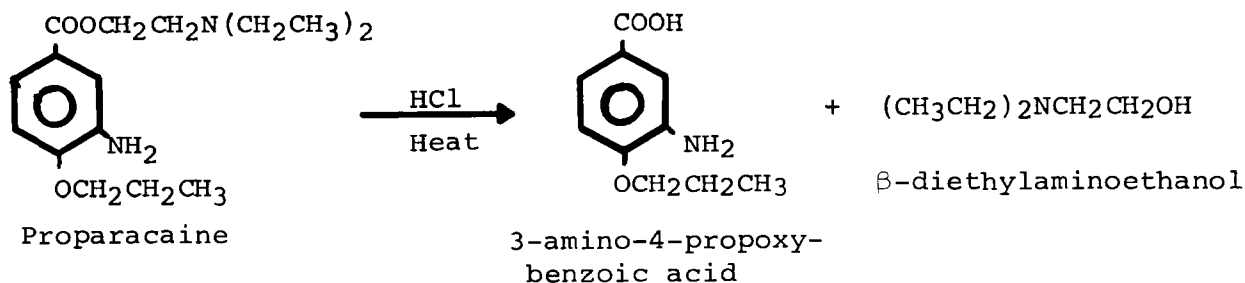


Figure 9. Hydrolysis of Proparacaine

5. Analysis of Intermediate Compound and Hydrolysis Products

Traces of the nitro intermediate (III in Figure 8) in proparacaine hydrochloride have been determined polarographically by Kocy⁴⁵. A Leeds and Northrup Electrochemograph type E equipped with a saturated calomel electrode and a dropping mercury electrode was used. The electrolyte buffer used is pH 4.0 acetate buffer containing 0.001M dodecyltrimethylammonium chloride (DTAC) as a maxima suppressor. The "standard addition technique" allows a quantitative method for determining as little as 0.1% of III in proparacaine hydrochloride. The nitro intermediate has an average reduction potential of -0.37 volts (vs. S.C.E.)

3-Amino-4-propoxy-benzoic acid, a hydrolysis product of proparacaine, has been separated from proparacaine by liquid-liquid extraction. The free acid remains in pH 6.8 buffer while proparacaine is extracted into chloroform²⁷. The aqueous layer is assayed spectrophotometrically for 3-amino-4-propoxy-benzoic acid. When the pH of the aqueous layer is lowered to 4, the free acid is extracted into chloroform²⁶. Solutions of the free acid were spotted on silica gel thin-layer plates and developed in two separate solvent systems: System I, acetone:benzene:chloroform(20:40:40); and System II, benzene:chloroform:acetic acid(20:80:10). The position of the free acid, relative to caffeine, was 0.7 in System I and 1.6 in System II.

Diethylaminoethanol, also a hydrolysis product of proparacaine, has been determined in plasma by a colorimetric method with methyl orange²⁸.

6. Methods of Analysis

6.1 Identification Tests

U.S.P. methods¹ include the characteristic ultraviolet spectrum of proparacaine hydrochloride (Section 2.13) for its identification. Infrared spectroscopy (Section 2.11) may be used to identify the drug. The primary aromatic amino group is identified by reacting with aqueous sodium nitrite, cooling the mixture, and then adding a solution of β -naphthol in sodium hydroxide. The scarlet-red precipitate formed does not dissolve upon addition of acetone¹. Thin-layer chromatography (Section 6.62) and paper chromatography (Section 6.61) have been utilized for identity purposes. Photomicrographs of proparacaine crystalline derivatives (Section 2.21) have been used as an adjunct to other physical methods for characterization. Formation of solid derivatives (Table 4) and the determination of the melting ranges and the infrared spectra of these derivatives provide further parameters for identification.

Table 4
Proparacaine Derivatives

<u>Derivative</u>	<u>Melting Range (°C)</u>	<u>Reference</u>
Chloroplatinate	195.5-198.5	10
Flavianate	162.0-163.0 (dec)	15
Methiodide	145.0-147.5	10
Picrate	122-124	11
Reineckate	138.0-140.0	10
Styphnate	151-158	10
Tetraphenyl- borate ^a	143-147	11
	131.5-132.0	10

^a Polymorphism has been suggested¹¹

6.2 Elemental Analysis (as $C_{16}H_{26}N_2O_3 \cdot HCl$)

<u>Element</u>	<u>% Theory</u>	<u>% Reported</u>	
		<u>Ref. 15</u>	<u>Ref. 6</u>
Carbon	58.08		58.04
Hydrogen	8.23		8.11
Nitrogen	8.47	8.56	8.54
Chlorine	10.71	10.88	10.85

Chlorides may be determined⁶ by reacting the sample with excess silver nitrate in the presence of nitrobenzene and nitric acid. The excess silver nitrate is titrated with potassium or ammonium thiocyanate using ferric ammonium sulfate as the indicator.

6.3 Spectrophotometric Analysis

6.31 Ultraviolet Spectrophotometric Analysis

Since proparacaine displays a high degree of absorption in the 220 to 320 nm range, ultraviolet spectroscopy (Section 2.13) provides a convenient means for its assay. In local anesthetic formulations, the presence of some vasoconstrictor agents, preservatives, and salts will not interfere if these materials either a) do not display absorption in this area or b) are diluted to the point where their absorption is negligible. In more complicated formulations proparacaine has been effectively separated prior to ultraviolet analysis by extraction from an alkaline medium into either ether¹ or chloroform¹¹.

In the presence of strong acids, the aromatic amine forms a positively charged ammonium ion and the peak due to the participation of the amino group in resonance is nullified (Figure 4). This observation has been utilized in determining propoxycaine in the presence of procaine³⁰. The same phenomenon could be applied to the determination of proparacaine in the presence of procaine.

6.32 Fluorescence Spectrophotometric Analysis

Although fluorometric procedures for the assay of proparacaine hydrochloride have not been reported, they should be feasible because the native fluorescence of proparacaine hydrochloride in 0.1N sodium hydroxide is sufficiently strong (Section 2.15).

6.4 Titrimetric Procedures

6.41 Nonaqueous Titration

Proparacaine hydrochloride can be titrated with good precision using acetic perchloric acid²⁹.

6.42 Titration with Sodium Nitrite

This assay has been described for propoxycaine³² which is an isomer of proparacaine. In this assay, the primary aromatic amine undergoes diazotization and the end-point is determined by starch-iodide paper external indicator. Ferrocypen solution, which has been used as an internal indicator for sodium nitrite titrations⁴⁶, may be used instead of the cumbersome external indicator. Although this titration has not been reported for proparacaine, the presence of a primary aromatic amino group in proparacaine suggests applicability of this titration.

6.43 Spectrophotometric Titration with Nitrous Acid³¹

In this titration, absorbance measurements are made during the titration of the primary aromatic amine with nitrous acid. The absorbance readings are dependent on the spectra of nitrous acid and the diazo derivative formed. In plotting the absorbances against the volume of titrant added, the intersection of straight lines of different slopes (prior to and after reaction completion) is the end-point. This titration has been applied to the determination of propoxycaine. Since it depends on the diazotization of the primary aromatic amine, this titration should be

applicable to the determination of proparacaine.

6.5 Colorimetric Methods

6.51 With Bratton-Marshall Reagent

The utility of the Bratton-Marshall reagent in the analysis of primary aromatic amines is well known. Application of this reagent to the analysis of proparacaine hydrochloride has been described by Poet³³.

Add 5 ml of 0.1N hydrochloric acid and 35 ml of distilled water to a 100 ml volumetric flask containing about 4 mg of proparacaine hydrochloride. Add 2 ml of 1% sodium nitrite, wait 2 minutes then add 10 ml of 0.5% ammonium sulfamate. After 3 minutes add 10 ml of 0.1% Bratton-Marshall reagent (N-1-naphthylethylenediamine hydrochloride) in 70% propylene glycol. Dilute to the mark with distilled water and measure the absorbance at 550 nm against a Reagent Blank.

6.52 With Sodium 1,2-Naphthoquinone-4-sulfonate

In this assay procedure, the yellow sodium 1,2-naphthoquinone-4-sulfonate, in the presence of alkali, reacts with the primary amine to yield a highly colored orange-red product. The excess yellow reagent is then bleached with sodium thiosulfate after making the solution slightly acidic with acetate buffer. This procedure has been applied to the assay of local anesthetics including propoxycaïne³⁴ and could be extended to the determination of proparacaine.

6.6 Chromatographic Procedures

6.61 Paper Chromatography

Koehler and Feldmann¹¹ described two paper chromatographic systems used in separating and identifying local anesthetics including proparacaine. The drugs are extracted from their dosage forms and then subjected to paper chromatographic analysis using Whatman No. 1 paper. In the solvent system butyl alcohol:hydrochloric

acid:water (30:5:35.5) the Rf for proparacaine is 0.45 and in the system butyl alcohol:acetic acid:water (40:10:50) the Rf is 0.79. To locate the spots, the dried strips are either viewed under an ultraviolet lamp in a dark room or sprayed with a modified Dragendorff reagent (acidified mixture of potassium iodide, bismuth subnitrate, and iodine in water). An alternative spray solution is an acidic solution of potassium permanganate in water.

In the general screening of nitrogeous bases, Clarke⁹ uses a solution of citric acid in a mixture of 130 ml of water and 870 ml of n-butanol as the solvent system. The Whatman paper No. 1 is pre-treated by dipping in a 5% solution of sodium dihydrogen citrate and drying at 25°C for one hour. In this system, proparacaine has an Rf of 0.52.

6.62 Thin-Layer Chromatography

Thin-layer chromatography using silica gel plates has been reported for proparacaine. Using a solvent system of strong ammonium hydroxide solution:methanol(3:200), the Rf of proparacaine is 0.59⁹ and its position relative to codeine is 1.8²⁶. The spots may be located by acidified iodoplatinate spray or by p-dimethylaminobenzaldehyde spray. Alternatively, the spots may be located by viewing under ultraviolet light.

Local anesthetics have been analyzed using thin-layer chromatography by Fuwa and co-workers³⁵. Separation of the drugs was effected on silica gel plates using the solvent system, benzene:acetone:ammonium hydroxide(80:20:1). The spots were identified by the Ehrlich (p-dimethylaminobenzaldehyde) reagent.

7. Analysis of Hydrolysis Products in Body Fluids and Tissues

Reed and Cravey²⁶ reported the determination of 3-amino-4-propoxybenzoic acid (A) in body fluids

and tissues. They prepared tungstic acid protein-free filtrates from blood, liver, kidney, and brain. Urine and gastric specimens did not undergo filtrate preparation. With each specimen, the pH was adjusted to 4 and extracted five times with chloroform. The organic phase was then extracted with 0.064N sodium hydroxide and the aqueous layer, containing A, was measured spectrophotometrically. Table 5 shows tissue concentrations found in a single case.

Table 5
Tissue Concentrations of Hydrolysis
Product as Equivalent Proparacaine

<u>Specimen</u>	<u>mg/100 ml or 100 g</u>
Blood	1.5
Brain	0.4
Lung	1.2
Liver	1.7
Kidney	1.6
Urine	None detected
Stomach	None detected

For further identification, the aqueous layer is acidified and back extracted into chloroform. The chloroform extract is evaporated and the residue is subjected to thin-layer chromatography (See Section 5).

It is speculated that the strong native fluorescence of proparacaine hydrochloride (Section 2.15) could provide a sensitive technique for its assay in body fluids and tissues.

8. Serum Protein Binding

Dastugue and co-workers³⁶ studied the binding of some drugs including proparacaine hydrochloride with bovine serum proteins. Proparacaine hydrochloride was dissolved in 5 ml of serum and dialyzed at 4°C against a phosphate-chloride buffer of pH 7.4 for 48 hours. The concentration of proparacaine in the dialyzate was determined by

measuring the ultraviolet absorbance at 268 nm. Table 6³⁶ tabulates the amount of protein-bound drug depending on the concentration of drug in the serum.

Table 6
Serum Protein Binding of Proparacaine
Hydrochloride

<u>Drug Concentration</u> <u>ug/ml serum</u>	<u>% Bound Drug</u>
25	46.4
50	33.6
100	26.4
200	21.9
300	19.4
400	19.6

9. Drug Metabolism

The pharmacology of proparacaine hydrochloride has been investigated by different workers^{37,38,39,47}. There is no evidence of blood level studies for proparacaine hydrochloride in the literature.

Proparacaine hydrochloride is rapidly hydrolyzed by guinea pig liver homogenates⁴⁰. At 37°C in the presence of 0.067M phosphate buffer (pH 7.2), 456 μ mole of drug is hydrolyzed per gram of fresh tissue per hour.

In a single case where a person purportedly inhaled about 500 mg of a white crystalline material purchased as "super-cocaine", Reed and Cravey²⁶ identified the material to be proparacaine hydrochloride. Working with this case, they reported the hydrolysis of proparacaine in body fluids. This observation, similar to the hydrolysis of proparacaine when heated in 2N hydrochloric acid (Figure 9), is typical of the metabolic pathway found with other amino alcohol ester type anesthetics^{41,42}. Hydrolysis is accelerated by enzymes in the liver, other tissues,

and plasma^{43,49}.

About 2 hours after the administration of the drug, Reed and Cravey found no proparacaine in samples of the blood, brain, lung, and urine. Some amounts of the hydrolysis product, 3-amino-4-propoxy-benzoic acid, were found in the blood, brain, lung, liver, and kidney.

In studying the fate of procaine in man, Brodie, Lief, and Poet²⁸ found that some diethylaminoethanol is excreted in the urine while some of it is further metabolized in the body. It is speculated that the diethylaminoethanol formed from the hydrolysis of proparacaine follows the same fate in man.

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PROPYLTHIOURACIL

Hassan Y. Aboul-Encin

CONTENTS
Analytical Profile - Propylthiouracil

1. Description
 - 1.1 Nomenclature
 - 1.11 Chemical Names
 - 1.12 Generic Name
 - 1.13 Trade Name
 - 1.2 Formulae
 - 1.21 Empirical
 - 1.22 Structural
 - 1.23 Wiswesser Line Notation
 - 1.3 Molecular Weight
 - 1.4 Elemental Composition
 - 1.5 Appearance, Color, Odor
 2. Physical Properties
 - 2.1 Crystal Properties
 - 2.11 Crystallinity
 - 2.12 X-ray diffraction
 - 2.13 Melting Range
 - 2.2 Solubility
 - 2.3 Identification
 - 2.4 Spectral Properties
 - 2.41 Ultraviolet Spectrum
 - 2.42 Infrared Spectrum
 - 2.43 Nuclear Magnetic Resonance Spectrum
 - 2.44 Mass Spectrum and Fragmentometry
 3. Synthesis
 4. Stability, Decomposition Production and Metal Complexes
 5. Metabolism
 6. Method of Analysis
 - 6.1 Titrimetric Methods
 - 6.11 Aqueous
 - 6.12 Non-Aqueous
 - 6.2 Colorimetric
 - 6.3 Ultraviolet Spectrophotometric
 - 6.4 Chromatographic Analysis
 - 6.41 Paper Chromatography
 - 6.42 Column Chromatography
 - 6.43 Thin Layer Chromatography
 - 6.44 Gas Chromatographic Analysis
- References
Acknowledgement

1. Description

1.1 Nomenclature

1.11 Chemical Names

2-Thio-4-oxo-6-propyl-1,3-pyrimidine
 2-Thio-6-propyl-1,3-pyrimidine-
 4-one
 1,2-Dihydro-6-propyl-2-thioxo-
 pyrimidin-4-one
 4-Hydroxy-2-mercapto-6-propylpyri-
 midine
 4-Oxo-6-propyl-2-thio-1,2,3,4-tetra-
 hydropyrimidine
 2,3-Dihydro-6-propyl-2-thioxo-4(1H)-
 pyrimidinone
 6-Propyl-2-thiouracil.

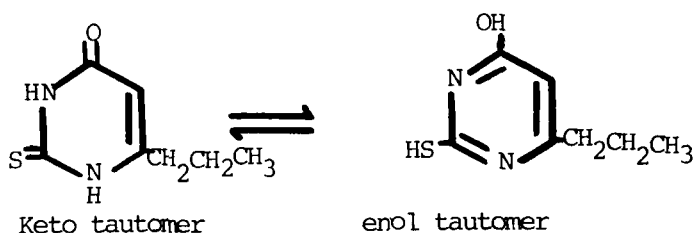
1.12 Generic Name: Propylthiouracil.

1.13 Trade Name: Propacil, Propycil,
 Prothyran, Procasil, Propyl-thyracil, Thyroestat II.

1.2 Formulae

1.21 Empirical: $C_7 H_{10} N_2 OS$

1.22 Structural:



1.23 Wiswesser Line Notation:
 $T_6 MYMVJ BUS F_3$

1.3 Molecular Weight: 170.23

1.4 Elemental Composition

C, 49.39%; H, 5.92%; N, 16.46%; O, 9.40%;
 S, 18.84%.

1.5 Appearance, Color, Odor:

White to pale cream-colored crystals or microcrystalline powder of starch-like appearance to the eye and to the touch; odorless; taste, bitter.

2. Physical Properties

2.1 Crystal Properties

2.11 Crystallinity

Propylthiouracil is a microcrystalline solid. Ashley (1) described a procedure for the preparation of distinctive crystals of propylthiouracil for the purpose of identification.

The crystals are prepared as follows:

Dissolve a few crystals of the sample in a drop of 0.1N NaOH on a slide, acidify by allowing a drop of 10% H₂SO₄ to coalesce gradually with the solution. ⁴Gently rock the slide to mix and examine microscopically. A typical photomicrograph of these crystals is shown in Fig.1.A. Furthermore, crystals are obtained by quickly smearing a drop of saturated solution of the sample in 75% alcohol at 70° over the whole surface of the slide with a small glass rod, and allowing the solvent to evaporate at room temperature. Photomicrograph of these crystals is shown in Fig.1.B.

Kassau (2) described the crystallinity of some pyrimidine derivatives including propylthiouracil by microsublimation.

2.12 X-ray Diffraction

Although the X-ray diffraction of propylthiouracil is not described in the literature. Nisi et al (3) described the elemental crystal structure for the reaction product between propylthiouracil and formaldehyde under acidic condition, 8-propyl 6H-pyrimido [2,1-d] [1,3,5] oxathiazin-6-one.

2.13 Melting Range

USP XIV(4) specifies a melting range for propylthiouracil between 219 - 221° as a criteria of acceptability.

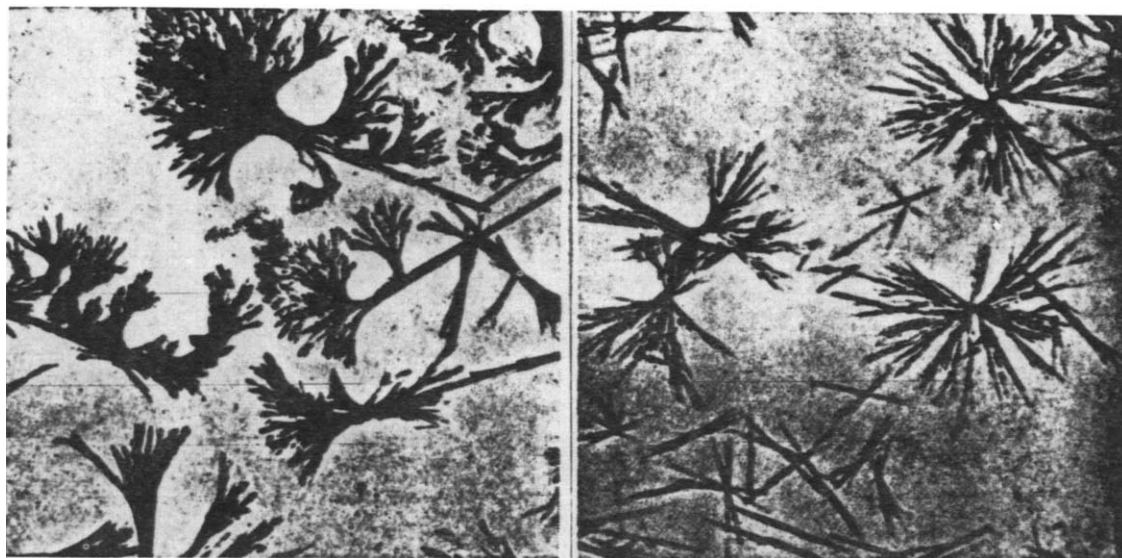


Fig. 1-A by acid precipitation

Fig.1-B from alcohol

Fig. 1 : Photomicrograph of propylthiouracil crystals.

Table I shows the melting range of propylthiouracil reported in the literature

Table I

m.p., C ^o	Reference
218-221	5
219-221	6
220	7
219	8
215-216	9

2.2 Solubility

Propylthiouracil is sparingly soluble in water (1:900 at 20^o); soluble in 100 parts boiling water, in 60 parts of ethanol; in 60 parts of acetone. Practically insoluble in ether, chloroform, benzene. Freely soluble in aqueous solutions of ammonia and alkali hydroxides. A saturated aqueous solution is neutral or slightly acidic to litmus.

2.3 Identification

The following identification tests are published in B.P. 1973(7) as a part of the identification of propylthiouracil. These tests are identical to the identification tests of methylthiouracil with the exception of the melting point.

(a) To a boiling saturated solution, add an equal volume of a freshly prepared solution containing 0.4% w/v of sodium nitroprusside, 0.4% w/v of hydroxylammonium chloride, and 0.8% w/v of sodium carbonate; a greenish blue color is produced.

(b) To 25 mg of propylthiouracil, add bromine solution drop by drop with completely dissolved, cool, and add 10 ml of barium hydroxide solution; a white precipitate is produced.

Bucher (10) introduced a modification to the above mentioned test in which excess bromine water was added then the excess bromine was removed by treating the solution until the solution was colorless and then the test was done as described before.

Metto and deFigueiredo (11) described a color test for thiouracil and its homologes as follows:

To a sample of the material add 0.5 ml 0.1 N NaOH and 10 ml of water; then introduce 10% CuSO_4 dropwise until an excess is present. Propylthiouracil gives a dark gray precipitate becoming bluish and then purplish gray.

Another color reagent was described by Nilsson (12) which can detect 1.3 mcg/ml of propylthiouracil in solution. Solutions required for the test were : 0.2 g 0-toulidine in 5% acetic acid, 1% CuCl_2 in water and 5% sodium acetate in water. A drop of each was mixed on a spot plate and a drop of propylthiouracil solution was added. An intensive blue color developed.

Propylthiouracil gives an orange-red color with 2,6-dichloroquinone chloroimide reagent which is sensitive enough to render the color test an excellent colorimetric analytical method of the drug in tablets which will be discussed later in the chapter (13).

The complex of propylthiouracil-chloroimide could be separated from chloroform solution as an orange-red needles (m.p. 172° with decomposition).

Bucher (10) reported a procedure for identification of thiouracil and its homologs through the preparation of their benzylthio ether derivatives (propylthiouracil benzylthio ether derivative m.p. $131-132^\circ$). p-Nitrobenzyl thio ether derivative (m.p. 193°) has been reported for propylthiouracil as a mean of identification of the drug (5).

2.4 Spectral Properties

2.4.1 Ultraviolet Spectrum

Propylthiouracil in neutral methanol absorbs ultraviolet radiation at 275nm (a_m 15800) and at 214 nm (a_m 15600) as shown in Fig 2A. In alkaline medium it shows 3 maxima at 315.5 nm (a_m 10900), 260 nm (a_m 10700) and at 207.5 (a_m 15400) as shown in Fig. 2B.

Galimberti et al (14) published a detailed study on the ultraviolet spectrophotometry of several derivatives. He reported that the replacement of an oxygen atom at C_2 by sulfur caused

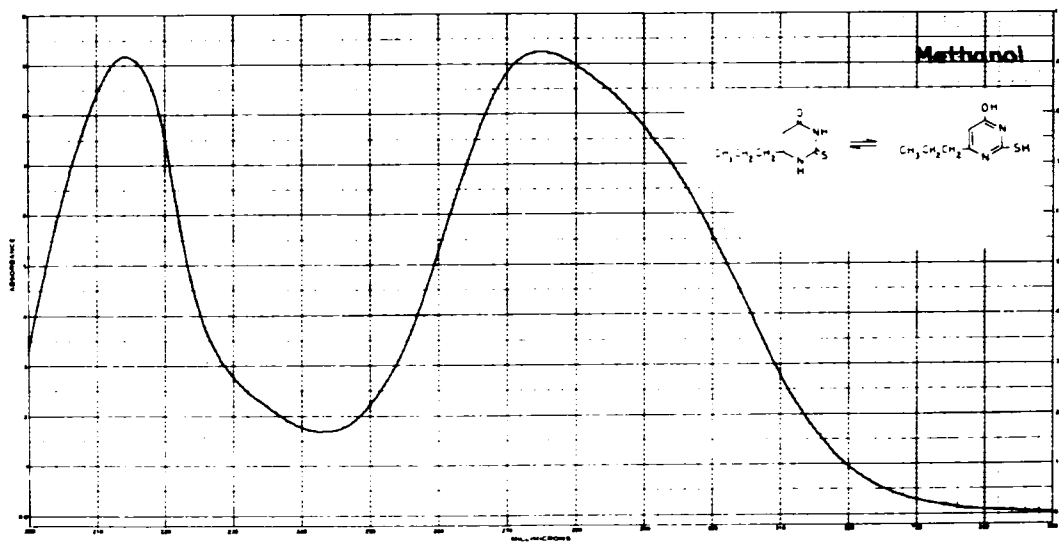


Fig.2-A: Ultraviolet spectrum of propylthiouracil in methanol.

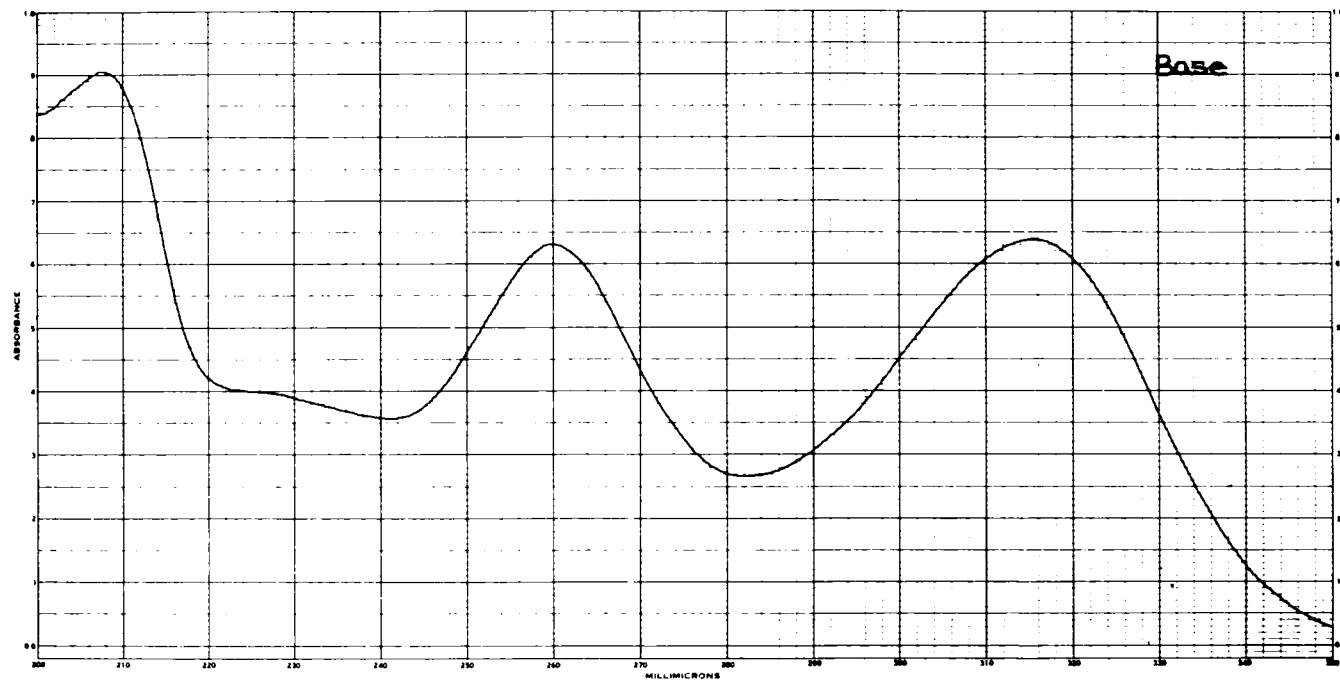


Fig.2-B: Ultraviolet spectrum of propylthiouracil in methanol potassium hydroxide.

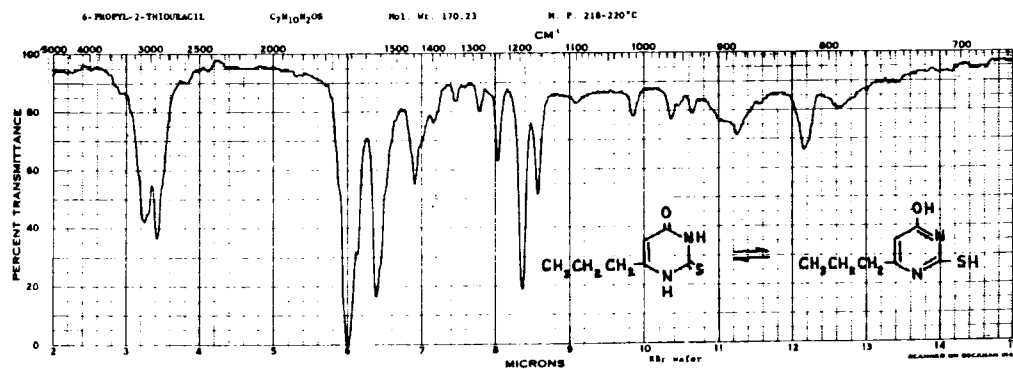
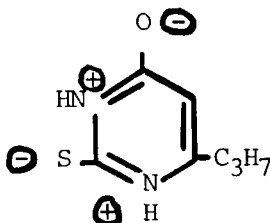


Fig. 3 : Infrared spectrum of propylthiouracil, KBr pellet.

a big shift to longer wavelength with increased absorption. Furthermore, the authors attributed the appearance of 3 maxima in case of the thiouracil and derivatives as compared to two maxima for the uracil homologs in alkaline medium (pH 11-12) to the double enolization. Informations with regard to the ultraviolet behavior of the drugs containing a thioamide-CONHCS - over a pH range from 1 to 13 were discussed by Stanovnik and Tisler (15). Their data indicated that the dipolar structure I was common with these compounds (Structure I for propylthiouracil at pH 7-8).



2.42 Infrared Spectrum

The infrared spectrum of propylthiouracil is shown in Fig 3. The spectrum was obtained on a Beckman IR4 spectrophotometer from KBr pellet.

The structural assignments have been correlated with the following band frequencies:

Frequency (cm ⁻¹)	Assignment
3120	NH Stretching
	imide
3020-2910	CH, CH ₂ , CH ₃
	stretching
2580 (weak since the Keto form predominates)	SH stretching
1650	C=O imide carbonyls

Other fingerprint bands characteristic to propylthiouracil are 1550, 1440, 1240, 1190, 1160, 880 and 810 cm^{-1}

Further information with regard to the infrared spectra of propylthiouracil is given in several references (8, 16).

2.43 Nuclear Magnetic Resonance Spectrum

A typical NMR spectrum of propylthiouracil is shown in Fig. 4. The sample was dissolved in deuterated dimethyl sulfoxide (DMSO- d_6). The spectrum was determined on a Varian T-60 NMR spectrometer with TMS as the internal standard.

The following structural assignments have been made for Fig. 4.

Chemical Shift (δ)	Assignment
Triplet at 0.93	$-\text{CH}_2\text{CH}_2\text{CH}_3$
Multiplet centered at 1.60	$-\text{CH}_2\text{CH}_2\text{CH}_3$
Multiplet centered at 2.50	$-\text{CH}_2\text{CH}_2\text{CH}_3$
(Solvent protons at 2.63 for DMSO- d_5).	
Singlet at 5.66	Olefinic proton at C_5
Broad singlet at 12.6	2-NH imide groups exchangeable with D_2O .

Further information concerning the interpretation of the NMR spectrum of propylthiouracil can be obtained from Sadtler NMR catalog(17) and also from CRC Atlas of spectral data (8).

2.44 Mass Spectrum and Fragmentometry

The mass spectrum of propylthiouracil obtained by conventional electron impact ionization shows a molecular ion M^+ at m/e 170. The M^+ ion peak has about 85% relative intensity (Fig. 5). The base peak is at m/e 68. The mass fragmentation mechanism of propylthiouracil is shown in Scheme I. It follows the same fragmentation pattern of uracil and derivative which has been established by several authors (18, 19, 20). It involves the loss of HCN_X ($X=\text{O}$ or S), and later verified by Hecht et al (21).

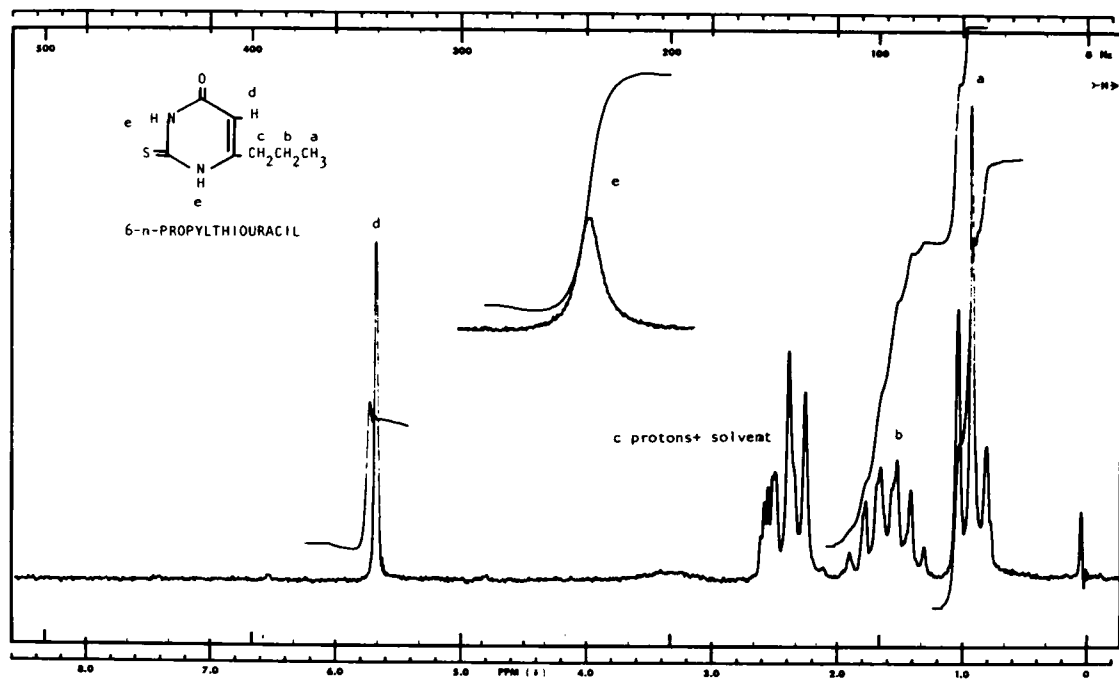


Fig. 4 : NMR Spectrum of propylthiouracil in DMSO-d₆ containing TMS as internal standard.

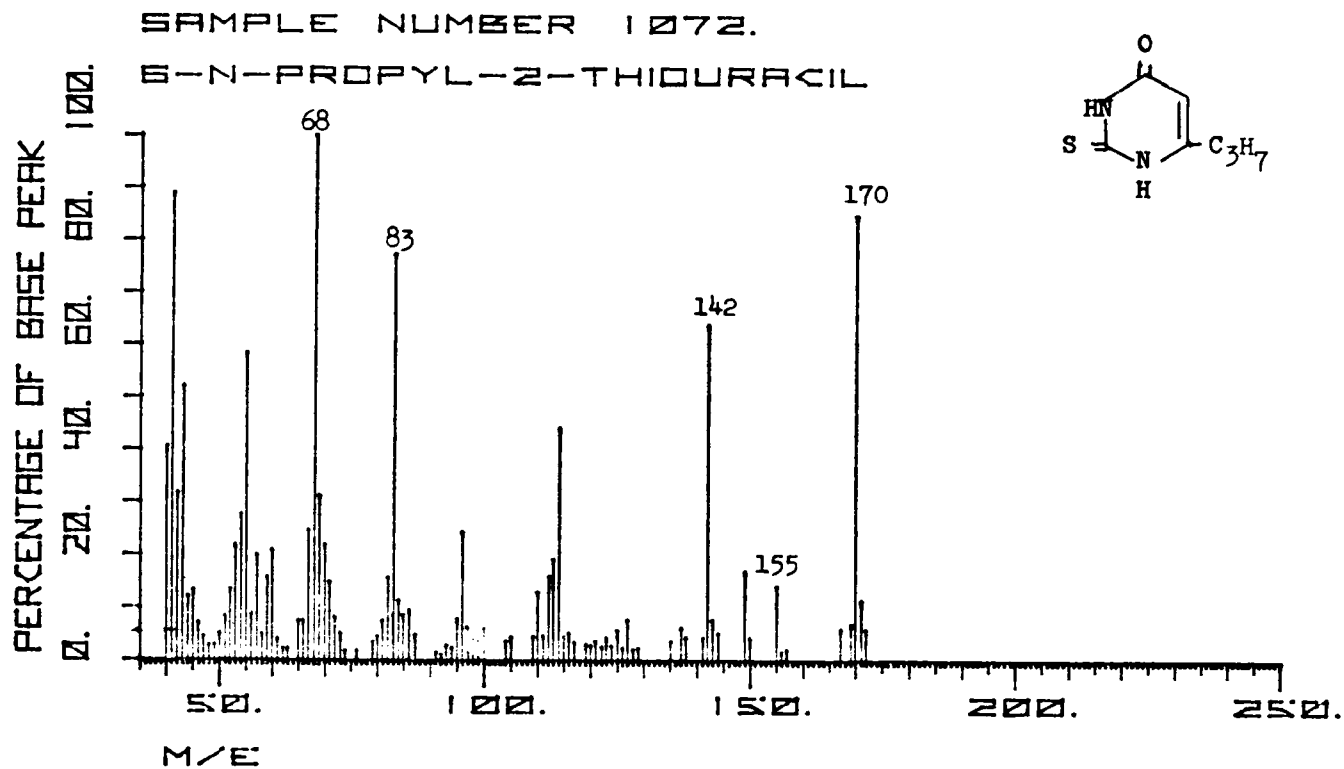
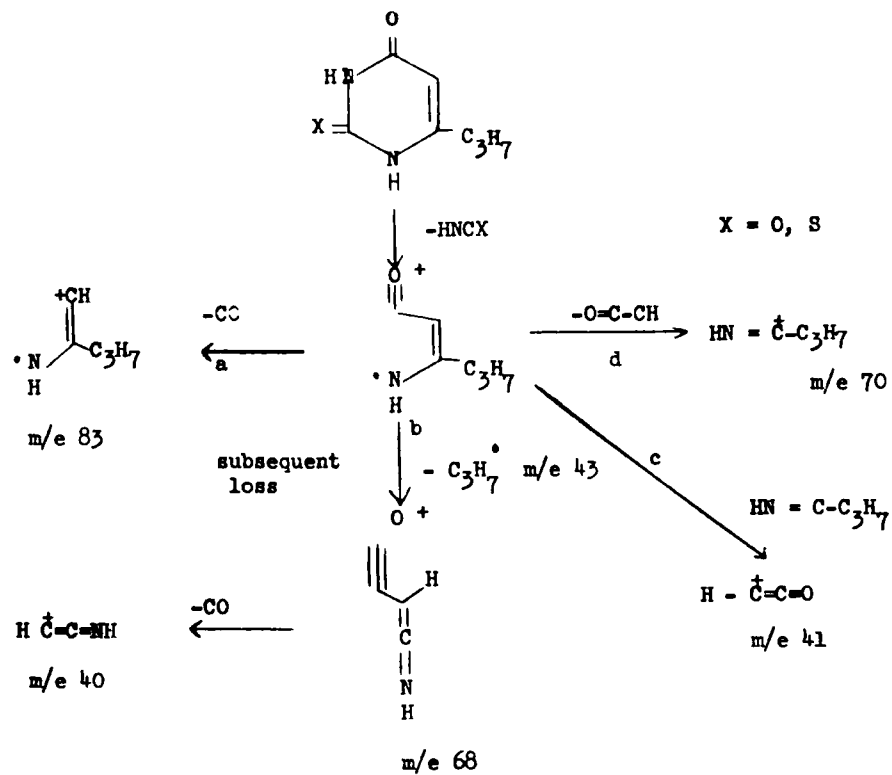


Fig. 5 : Mass Spectrum of propylthiouracil (EI).



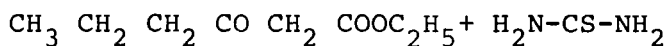
Scheme I

Mass Spectral fragmentation mechanism of Propylthiouracil (18,19,20,21).

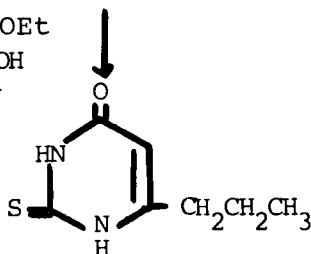
The first step in the fragmentation is a retro-Diel-Alder decomposition with a loss of HCNS and the production of ion radical which is only of minor importance since it immediately undergoes the following paths :

- (a) A loss of CO to give an abundant ion at m/e 83,
- (b) A loss of propyl radical to give an abundant ion at m/e 68, which subsequently base CO to give an ion at m/e 40.
- (c) A loss of $\text{HN}=\text{C}-\text{CH}_3$ ion to give ketene m/e 41.
- (d) A loss of $\text{CH}=\text{C}=\text{O}$ to give an ion m/e 70.

3. Synthesis



1) NaOEt
EtOH
2) H^+



Propylthiouracil is prepared by the condensation of ethyl 3-oxocaproate with dry thiourea in the presence of a base (22).

Several authors had modified the above synthetic procedure for patent purposes yet the principle still the same (9, 23, 24, 25).

4. Stability, Decomposition Product and Metal Complexes:

Propylthiouracil is a relatively stable compound at room temperature.

It is recommended that it should be kept in a well-closed containers protected from light.

Propylthiouracil forms metal complexes with divalent metals e.g., Cu^{+2} , Pb^{+2} , Cd^{+2} , Ni^{+2} , and Zn^{+2} but not with Fe^{+3} , Fe^{+2} , Co^{+2} , Ca^{+2} or Mn^{+2} . Garret and Weber (26, 27) published detailed studies on these metal complexes of thiouracil and analogs regarding their structures, stability constants, solubility analyses and spectrophotometric properties.

5. Metabolism

Interest in the metabolism of antithyroid drugs has recently been focused on 6-propyl-2-thiouracil, one of the current drug of choice in the treatment of hyperthyroidism. Propylthiouracil is readily metabolized after administration to humans and rats and the major metabolite in urine, plasma and bile has been identified as propylthiouracil glucuronide (28, 29, 30, 31, 32, 33).

Other metabolites identified in rat bile and urine are shown in Fig. 6. These include :

S-methyl-6-propylthiouracil (minor metabolite)	
6-Propyluracil (minor metabolite)	
6-propylthiouracil sulfenic acid] Identified in rat thyroid extracts
6-propylthiouracil sulfonic acid	
6-propylthiouracil sulfate	

Desbarats-Schönbaum et al (34) reported that in highly alkalinized guinea pig urine, propylthiouracil disulfide was isolated. The sulfur group of propylthiouracil appears to be the major site of alteration, biotransformation at this site results in a total or major loss of antiperoxidase activity (35). None of the metabolites isolated and identified was as active as the parent compound (35). Several metabolites of propylthiouracil remain unidentified.

It has been reported that the plasma half-life in hours of methimazole (another drug of choice in treatment of hyperthyroidism) was 2-5 times that of propylthiouracil (36).

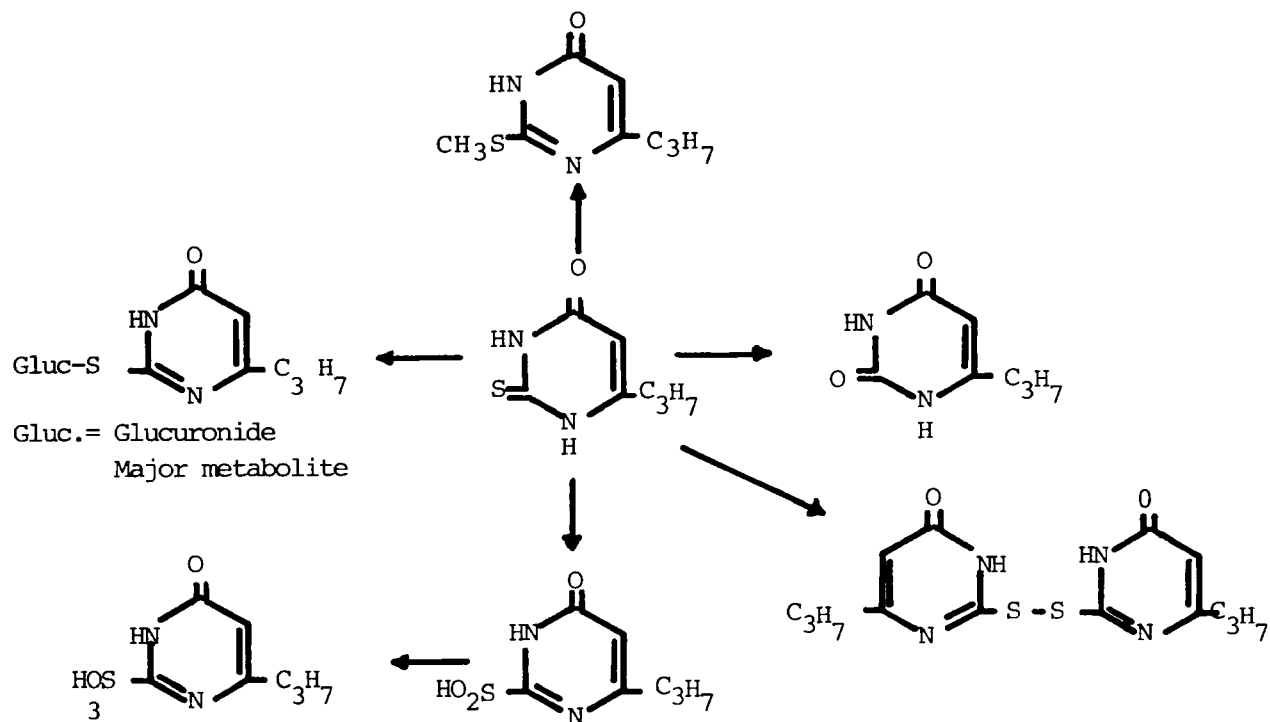


Fig.6 Metabolism of Propylthiouracil

6. Method of Analysis

6.1. Titrimetric Methods

6.1.1. Aqueous

Several titrimetric methods were developed for analysis of propylthiouracil.

1. Simple titration with standard NaOH, in neutral alcohol solutions, the N.N.R method. This method is simple. Both phenolphthalein or thymolphthalein being used as indicator. Yet the presence of stearic acid interferes with the assay and it should be removed by extraction with petroleum ether before titration (37).

2. Silver nitrate method: Berggren and Kirsten (38) introduced a modification to the above mentioned method. Acetone was used to extract propylthiouracil from most of the tablet excipients. Acetone extract was neutralized by adding HNO_3 or 0.1 N NaOH using 1, 2, 5-dinitrophenol as indicator. To the neutralized solution, water was added and a certain volume of 0.1 N AgNO_3 was added and the solution was titrated with 0.1 N NaOH to a persisting blue color (bromothymol blue was used as indicator). This method was found satisfactory yet if stearic acid was present, it should be removed before addition of AgNO_3 . It was adopted by USP XVIII.

3. Mercuric acetate titrimetric method: Abbot (39) described a method for determination of thiouracil and analogs by titrating the solution with 0.05M $\text{Hg}(\text{OAc})_2$ using 0.5% diphenylcarbazone in ethanol as indicator. The method was adopted by B.P. 1973 and USP XIV issue because excipients of starch, sucrose, acacia, rosin, calcium carbonate, stearic acid or magnesium stearate did not interfere.

4. Potassium Bromate titrimetric method: The bromometric method was developed by Wojahn and Wempe (40, 41, 42) and was reported to be more satisfactory and accurate method than USP XIV procedure using mercuric acetate method, since the presence of lactose in propylthiouracil tablets interfered with the mercuric salt method. To an

alkaline solution of propylthiouracil bromination was effected by 0.1 N KBrO_3 and KBr in presence of 25% HCl. After one hour, an excess of 0.1 N NaAsO_2 was added and back-titrated with 0.1N KBrO_3 with ^2p -ethoxychrysoidine as the indicator.

6.12 Non Aqueous

Backe-Hansen (43) reported a non-aqueous titration method for propylthiouracil using sodium methoxide in benzene and methanol in a solution of dimethylformamide or pyridine (against thymol blue or azoviolet as indicator). Lithium methoxide 0.1 N in benzene and methanol had also been used instead of sodium methoxide (5).

6.2. Colorimetric

A number of colorimetric analytical methods had been developed for the determination of propylthiouracil in pure form, pharmaceutical formulations, tablets and animal feeds.

(a) The use of Grote reagent:

Doden et al (44) had applied the use of Grote's reagents to determine different thiouracils quantitatively. The absorption maxima at 660 nm was measured. The reaction obeyed Beer's Law over the concentration range 0.5×10^{-4} to 3×10^{-4} M.

Brueggeman and Schole (45) applied the color reaction of Grote reagent with thiouracils for the quantitative determination of thiouracils in feeds. Bucci and Cusmano (46) reported a similar colorimetric method using Grote's reagent as modified by Christeinsen (47). The authors claimed that the method was suitable for the analysis of thiouracil in very small amounts (200 p.p.m) in the presence of other biological substances.

(b) 2,6-Dichloroquinone Chloroimide reagent:

McAllister and Howells (13) reported a method for analysis of propylthiouracil in tablets using 0.4% solution of 2,6-dichloroquinone chloroimide in aldehyde-free absolute ethanol. The yellow color obtained from such reaction was extracted in chloroform and optical density of the solution was compared with a standard graph.

(c) Ruthenium chloride color reaction:
Reinhardt (48) described a colorimetric method for determination of propylthiouracil and similar compounds. It was based on the reaction between the thiocarbamide linkage - CONHCS - and RuCl_3 in strong acid medium. The color developed was measured at 520 nm and compared with a standard curve.

(d) Isopropylamine - cobalt acetate reagent:
Holt and Mattson (49) developed a colorimetric assay for compounds containing the groups - CONHCO - and - CONHCS - , including propylthiouracil. A color developed with the reaction of these compounds with isopropylamine reagent (50 ml of the amine made to 200 ml with dry chloroform) and cobalt acetate (made of 0.25g in 200 ml methanol). The color was measured at 530 nm and compared with standards. The method was sensitive to a concentration of 1 mcg/ml.

(e) Hydroxylamine hydrochloride - Sodium nitroprusside reagent:
Doden and Kopf (50) published a colorimetric method for the determination of thiouracil and analogs using hydroxylamine hydrochloride and sodium nitroprusside in the presence of sodium bicarbonate, bromine and phenol. The greenish blue color developed was compared with standard curve.

(f) Potassium iodate - acetic acid color reaction:
 A method based on the color developed by the reaction of propylthiouracil and its methyl analog, with KIO_3 and acetic acid was used to determine these drugs in tablets (51).

Powdered tablets containing approximately 50 mgs, in 10 ml ethanol and 30 ml of water, were allowed to stand for 30 minutes. The filtered solution (0.5 ml), 5 ml of KIO_3 (0.5 w/v% solution), and 2 ml of acetic acid were diluted with water. The color at 465 nm was measured after 80 minutes. The solutions were stable for a further 30 minutes. A calibration curve was made for comparison.

6.3. Ultraviolet Spectrophotometric:

The alkaline solution of propylthiouracil shows two peaks, one at 234 nm and the other at 260 nm, the first maximum has a higher molar absorptivity. The ultraviolet absorption of propylthiouracil in ammoniacal solution at 234 nm is used as a sensitive criteria for its analysis in pure and tablet forms (52, 53, 54). This method is sensitive to a concentration of 7.5 mcg/ml and satisfactory results are obtained. Yet, Bruggeman and Schole (45) used the absorption maximum at 260 nm for the analysis of propylthiouracil in feeds.

6.4. Chromatographic Analysis:

6.41. Paper:

The chromatographic behavior of propylthiouracil and related analogs were discussed by several authors (55, 56, 57), for the purpose of separation and identification in biological fluids and pharmaceutical preparations. Table II summarizes the solvent systems and visualizing agents used.

Table II

<u>Solvent System</u>	<u>Visualising agent</u>	<u>Reference</u>
C ₆ H ₆ :EtOH 16:6	RuCl ₃	55
AmOH : H ₂ O	I ₂ vapor or dichlorobenzoquinone chloroimide and alkali	56

6.42. Column Chromatography:

Lindsay *et al* (32, 33) separated and purified propylthiouracil from its S-methyl derivative and other metabolites using column chromatography on Bio-Gel P-2 columns (200-400 mesh) with water and on DEAE-Sephadex A-25 columns eluting with freshly prepared 0.1 M ammonium acetate.

6.43. Thin Layer Chromatography:

Begliomini *et al* (58) described a procedure for the separation and identification of several antithyroid drugs including propylthiouracil

in animal feeds and biological samples by means of tlc on silica gel G. The solvent system was a mixture of 50 ml chloroform, 6 ml isopropanol, and 0.1 ml glacial acetic acid. Amounts up to 1 mcg were detected by this method. Propylthiouracil showed R_f value of 0.81 while its methyl homologs moved slower R_f 0.65.

Other solvent systems used to identify propylthiouracil and its metabolites and derivatives were published by Lindsay *et al* (32, 35) and summarized in Table III.

Table III

<u>Solvent System</u>	<u>Developer</u>
0.05 M Ammonium acetate	uv
1 M Ammonium acetate ethanol	
15:75	uv
C ₆ H ₆ : isopropanol 6:1	uv
Hexane : acetone : ethanol	
60:20:2	uv
Hexane : acetone 3:1	uv

6.44. Gas Chromatographic Analysis:

Although a number of methods are available for the determination of propylthiouracil and its analogs, yet all these methods are largely based on the properties of the sulfhydryl groups (-SH). However, the same properties are also common to the C=S and -S-S- groups.

Fravolini and Begliomini (59) developed a simple, rapid gas chromatographic method for determination of thiouracils in animal feeds. The method was selective and sensitive (able to detect 0.1 mcg of thiouracils). The chromatographic separation was carried out on dialkylated thiouracils prepared according to Wheeler (60). The best results were obtained with a glass column containing Chromosorb as a solid support and 3% SE-30 polymer methyl silicone as the liquid phase. The procedure permitted simultaneous identification and determination of a variety of thyrostatic products. A typical chromatogram is shown in Fig.7

Retention times were similar to those reported by other authors (61) except that the 5-methyl and 6-methyl thiouracils eluted in the order shown in Table IV.

Table IV

Retention Times of Thioracils

Compounds	Retention times, Sec.
2-Thiouracil	160
5-Methyl-2-thiouracil	178
6-Methyl-2-thiouracil	190
6-Propyl-2-thiouracil	322
6-Phenyl-2-thiouracil	774

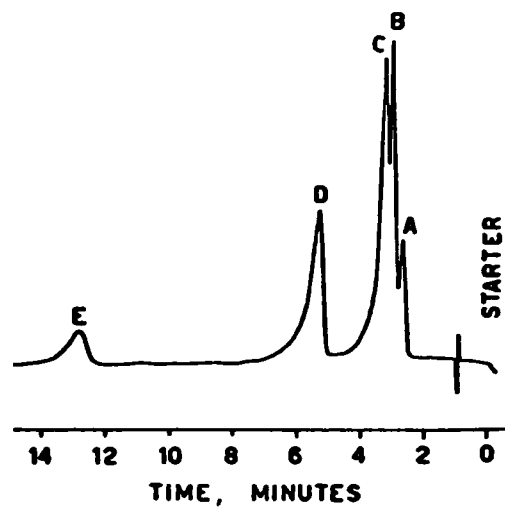


Fig. 7: Gas Chromatograph of thiouracils.
A = 2-thiouracil B = 5-methyl-2-thiouracil
C = 6-methyl-2-thiouracil D = 6-propyl-2-thiouracil
E = 6-phenyl-2-thiouracil.

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SODIUM NITROPRUSSIDE

Richard Rucki

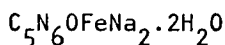
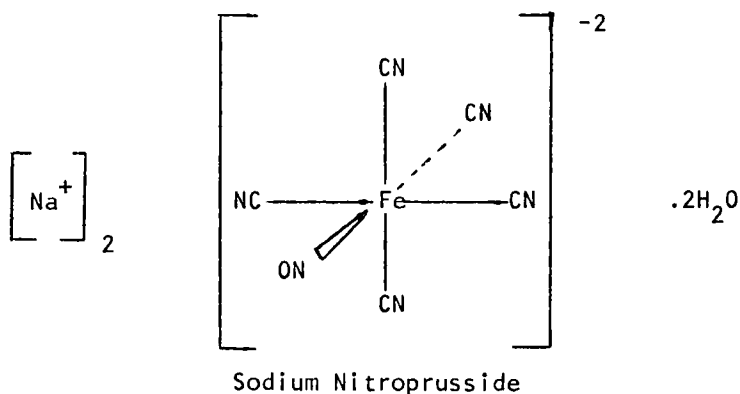
INDEX

1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor
2. Physical Properties
 - 2.1 Infrared Spectrum
 - 2.2 Raman Spectroscopy
 - 2.3 Ultraviolet/Visible Spectrum
 - 2.4 Fluorescence Spectrum
 - 2.5 Optical Rotation
 - 2.6 Differential Scanning Calorimetry
 - 2.7 Thermogravimetric Analysis
 - 2.8 Solubility
 - 2.9 Crystal Properties
 - 2.9.1 Crystal Structure
 - 2.9.2 X-Ray Diffraction
3. Synthesis
4. Stability and Degradation
 - 4.1 Solid Stability
 - 4.2 Stability in Solution
5. Drug Metabolic Products
6. Toxicity
7. Methods of Analysis
 - 7.1 Elemental Analysis
 - 7.2 Identification Tests
 - 7.3 Thin-Layer Chromatographic Analysis
 - 7.4 Spectrophotometric Analysis
 - 7.5 Colorimetric Analysis
 - 7.6 Polarographic Analysis
 - 7.7 Coulometric Analysis
 - 7.8 Titrimetric Analysis
 - 7.9 Miscellaneous Methods of Analysis
8. Acknowledgements
9. References

1. Description

1.1 Name, Formula, Molecular Weight

Sodium nitroprusside is disodium pentacyanonitrosylferrate (2-) dihydrate. It is also known as sodium nitroferricyanide and sodium nitroprussiate. The dihydrate is the common form of the compound and is assumed in this report except where specified as anhydrous.



Molecular Weight:
297.95

1.2 Appearance, Color, Odor

Red-brown, practically odorless, crystals or powder.

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of sodium nitroprusside is presented in Figure 1 (1). The spectrum was recorded on a Perkin-Elmer Model 621 Grating Infrared Spectrophotometer (Survey Conditions). The sample was dispersed in Fluorolube^R to record the spectrum in the region of 4000-1340 cm^{-1} and in mineral oil for the region of 1340-370 cm^{-1} . Assignments for the bands in Figure 1 are listed in Table I (1). These assignments are in agreement with those reported in the literature (2-4).

FIGURE 1
Infrared Spectrum of Sodium
Nitroprusside

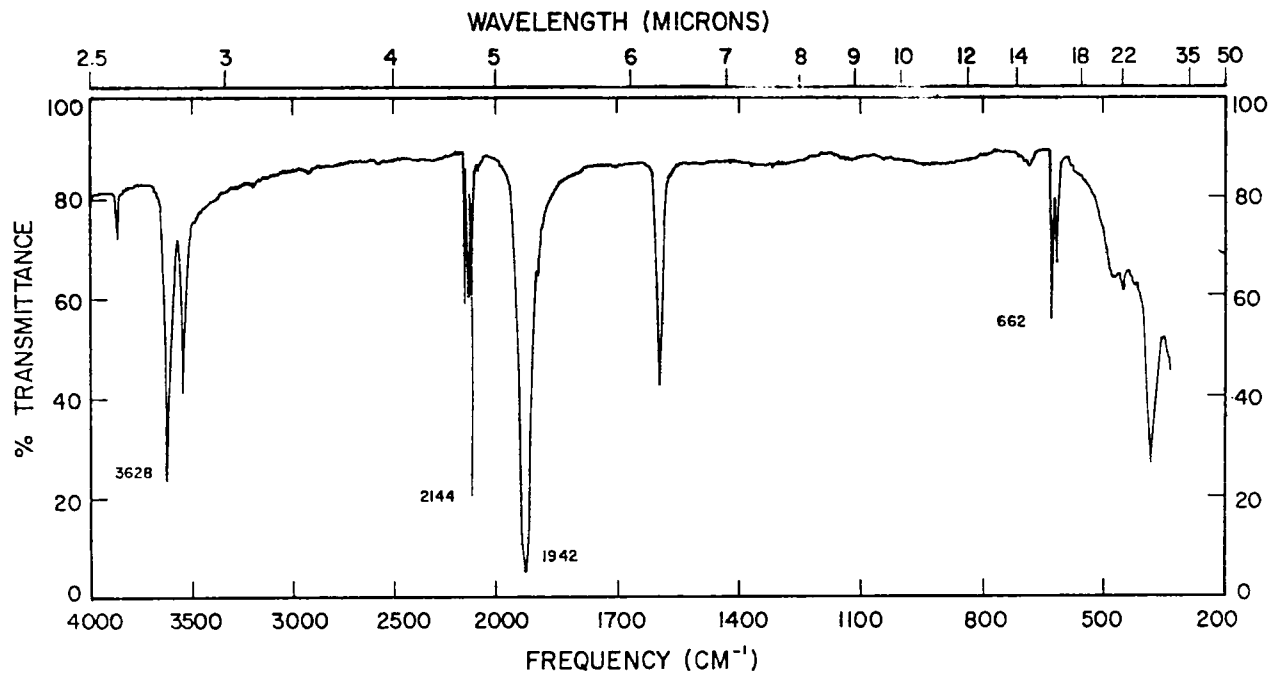


TABLE I
Infrared Assignments for Sodium Nitroprusside

<u>Band (cm^{-1})</u>	<u>Assignment</u>
3628	Asymmetric OH stretch
3547	Symmetric OH stretch
2174	$\text{-C}\equiv\text{N}$ axial stretch
2144, 2157, 2162	$\text{-C}\equiv\text{N}$ radial stretch
1942	$\text{N}\rightarrow\text{O}$ stretch
1614, 1618.5, 1624	OH bending
662	$\text{Fe-N}\rightarrow\text{O}$ linear bending
651	Fe-N stretch
491	$\text{Fe-C}\equiv\text{N}$ bending
461	$\text{Fe-C}\equiv$ axial stretch
418.5	$\text{Fe-C}\equiv\text{N}$ bending

2.2 Raman Spectroscopy

Raman spectra of single-crystal sodium nitroprusside have been utilized for structure elucidation by a number of investigators (2, 5-8). The use of a He-Ne laser to excite the oriented crystal has been reported (2,8). Polycrystal sodium nitroprusside rapidly oxidized when subjected to laser excitation (9).

2.3 Ultraviolet/Visible Spectrum

The ultraviolet/visible spectrum of sodium nitroprusside (750 mg of sodium nitroprusside/100 ml of water vs. water in the reference cell) in the region of 240 to 700 nm exhibits two maxima at 390-395 nm (molar absorptivity, $\epsilon = 20.4$) and at about 500 nm (appears as a shoulder). The instrument used was a Cary 14 Recording Spectrophotometer. The visible portion of the spectrum is shown in Figure 2 (10). These results are in agreement with UV/visible data reported previously in the literature (11-13). The existence of the maximum at 500 nm as a distinct absorption band (a distinct electronic transition) has been confirmed by the determination of the polarized crystal spectrum of a single crystal of sodium nitroprusside dihydrate (12).

2.4 Fluorescence Spectrum

Sodium nitroprusside exhibits no fluorescence in acidic, basic or neutral media (14).

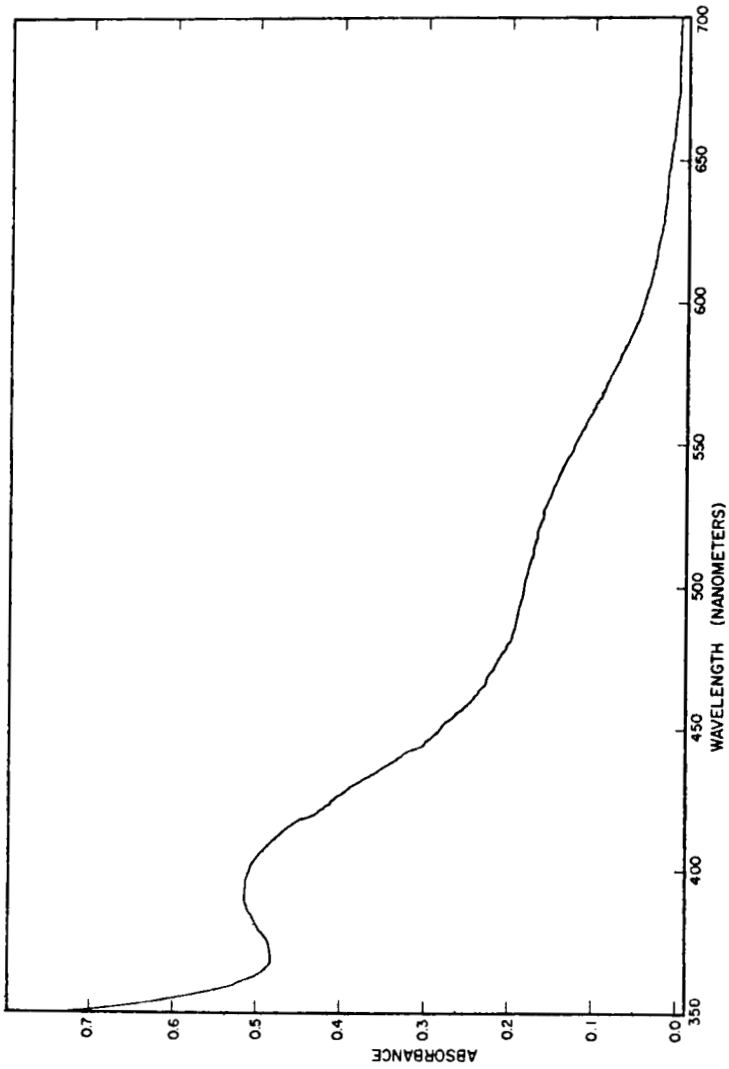
2.5 Optical Rotation

A 0.6% (w/v) solution of sodium nitroprusside in water exhibited no optical rotation between 650 and 220 nm (15).

2.6 Differential Scanning Calorimetry

DSC scans for typical lots of sodium nitroprusside at a scan rate of 20°C/minute exhibit two very broad endotherms, the first between about 125 and 180°C and the second between about 320 and 360°C, followed immediately by an exotherm. The endotherms do not correspond to sample melt and have the typical appearance of volatile material leaving the system. Anhydrous sodium nitroprusside did not exhibit the

FIGURE 2
Visible Absorption Spectrum of Sodium
Nitroprusside



first endotherm (16). The temperature of each endotherm corresponds to a weight loss in the TGA (Section 2.7). Thermal analysis of sodium nitroprusside has been reported in the literature (17-19).

2.7 Thermogravimetric Analysis

TGA scans for typical lots of sodium nitroprusside exhibit two discrete weight losses. The first occurs between 100 and 190°C and accounts for 12 to 13% of sample weight (theoretical weight loss for dihydrate is 12.09%). The second occurs between about 280 and 390°C and accounts for 17.6 to 19.9% of sample weight (theoretical weight loss for cyanogen, $(\text{CN})_2$, is 19.85% of anhydrous sample weight) (16). The identification of the second weight loss as cyanogen is speculative. Chamberlain and Greene (17,18), using dynamic gas evolution analysis, have reported that the thermal decomposition of cyanonitrosyl ferrates involve the evolution of water, $(\text{CN})_2$ and NO . Gentil et al. (19) and Mohai (20,21) have reported TGA data for sodium nitroprusside.

2.8 Solubility

Approximate solubility data obtained for a sample of sodium nitroprusside at 25°C are listed in Table II (22). Equilibration time was 20 hours for each system.

TABLE II

Solubility of Sodium Nitroprusside

<u>Solvent</u>	<u>Solubility (mg/ml)</u>
Water	>200
95% Ethanol	1.1
Absolute Ethanol	5.0
Methanol	100-200
Acetone	Insoluble (<0.05)
Diethyl Ether	Insoluble (<0.05)
Chloroform	Insoluble (<0.05)
Benzene	0.2
Isopropyl Alcohol	0.1
Hexane	0.1
Ethyl Acetate	0.3
Normal Saline	>200

2.9 Crystal Properties

2.9.1 Crystal Structure

Sodium nitroprusside occurs as reddish-brown (or ruby-red) crystals; anhydrous (lyophilized) sodium nitroprusside exists as a light orange, uniform powder (23).

The crystal structure of sodium nitroprusside has been studied via X-ray diffraction, infrared and Raman analysis (2-4, 24-27). The crystal is orthorhombic with space group D_{2h}^{12} -Pnnm. The nitroprusside ion lies on the $2b$ mirror plane and has approximately C_{4v} symmetry. The unit cell contains four formula units of the type $Na_2Fe(CN)_5NO \cdot 2H_2O$. The crystal structure is composed of Na^+ , $Fe(CN)_5NO^{2-}$, and H_2O units. The $Fe(CN)_5NO^{2-}$ ions occupy sites of C_s symmetry, and the H_2O molecules occupy C_1 sites. The ligands are colinear with the

metal atom, which is displaced slightly in the direction of the NO group from the plane of the four pseudo-equivalent CN groups. Each sodium ion lies at the center of a distorted octahedron composed of four CN groups and two water molecules. These octahedra share edges in such a way that each CN group is coordinated to two Na⁺ ions, as is each water molecule. The nitroso group is coordinated only to Fe⁺² (24). The water molecules are not involved in any significant hydrogen bonding with the Fe(CN)₅NO²⁻ ion; they merely serve to fill the empty space in the lattice (2, 24, 26). Studies of the infrared (28, 29) and Mössbauer (30) spectra indicate a large amount of back bonding between the metal and the nitrosyl ligand. Although the formal charge of iron and nitrosyl in the complex has been a matter of controversy, the general consensus appears to be that Fe and NO have formal charges of +2 and +1, respectively (12, 24, 31-35).

2.9.2 X-Ray Diffraction

The X-ray powder diffraction pattern of a proposed house standard of sodium nitroprusside is presented in Table III (36). The interplanar spacings agree with those reported in the literature using a molybdenum target (37).

Instrumental Conditions

Instrument	GE Model XRD-6 Spectrogoniometer
Generator	50 KV, 12.5 mA
Tube Target	Copper (Cu K α = 1.5418Å)
Optics	0.1° Detector slit M.R. Soller slit 3° Beam slit Ni Filter 4° Take-off angle
Goniometer	Scan at 0.4° 2 θ /minute
Detector	Sealed proportional counter 1.75 KV (front), 0.95 KV (rear). Pulse height selector El 5

volts, window out.
 Time constant = 2.5 seconds
 Range = 1000 c/sec full
 scale
 Recorder Synchronized with gonio-
 meter at 1"/2.5 minutes
 Sample Ground at room temperature

TABLE III

Sodium Nitroprusside

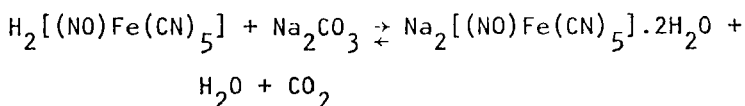
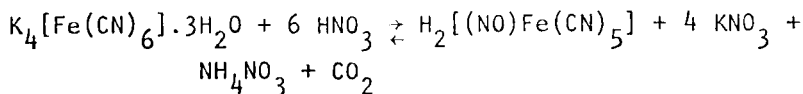
<u>2θ</u>	<u>d(\AA)[*]</u>	<u>I/I₀^{**}</u>
11.60	7.63	21
15.61	5.68	55
19.00	4.67	58
21.65	4.10	98
23.08	3.85	29
27.14	3.29	34
31.25	2.86	100
33.30	2.69	16
35.65	2.52	25

* d (interplanar distance) = $n\lambda/2 \sin \theta$

** I/I₀ = relative intensity (highest
 intensity = 100)

3. Synthesis

Sodium nitroprusside is commonly prepared by the oxidation of potassium ferrocyanide with dilute nitric acid and subsequent neutralization of the liquid with sodium carbonate (38). The reaction scheme is shown below (39):



4. Stability and Degradation

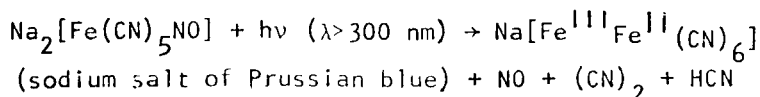
4.1 Solid Stability

Sodium nitroprusside crystals have been reported to be stable in air (40). Even in the dry, solid state, however, the compound is somewhat light sensitive (Section 4.2) and should be protected from light (41, 42). Small amounts of moisture could facilitate the photodegradation of dry sodium nitroprusside (41). In closed, amber vials at 25°C, sodium nitroprusside in the solid state remains suitably stable for at least 24 months (measuring absorbance maximum at 394 nm; Section 4.2) (43).

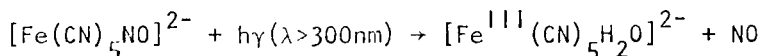
4.2 Stability in Solution

Sodium nitroprusside in solution is extremely photosensitive, undergoing rapid and numerous reactions, many of which are undefined. Literature descriptions of the photodecomposition products of nitroprusside are, in some cases, contradictory.

In direct sunlight $[\text{Fe}(\text{CN})_5\text{NO}]^{2-}$ ultimately yields Prussian blue, HCN and NO (44). Kapatos et al. (45) have reported that photoirradiation of solutions of nitroprusside yield Prussian blue and NO, while Wolfe and Swinehart (46) report a similar reaction in unbuffered solutions of nitroprusside:

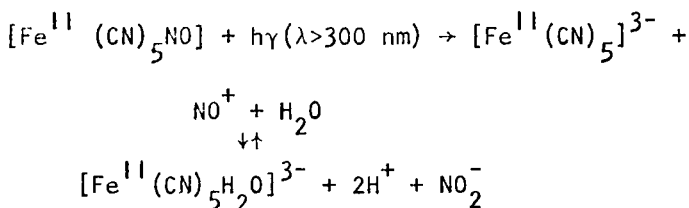


In solutions buffered at pH 6, Wolfe and Swinehart (46) have reported formation of the pentacyanoaquo-ferrate (III), agreeing with several other papers (47-50):



The pentacyanoaquo-ferrate(III) undergoes rapid equilibrium with $[\text{Fe}_2^{\text{III}}(\text{CN})_{10}]^{4-}$ (50, 51).

Photoaquation to yield $[\text{Fe}^{\text{II}}(\text{CN})_5\text{H}_2\text{O}]^{3-}$ and NO has been described most frequently as the primary photochemical reaction of nitroprusside (11,34,52-54). Mitra and coworkers (34) found a pH decrease upon photolysis and attributed this to hydrolysis of the nitrosyl cation:



The pentacyanoaquo-ferrate (II) undergoes rapid equilibrium with $[\text{Fe}_2(\text{CN})_{10}]^{6-}$ (55).

Photoreduction of $[\text{Fe}(\text{CN})_5\text{NO}]^{2-}$ to $[\text{Fe}(\text{CN})_5]^{3-}$ in aqueous solution has been reported (56). The orange-to-blue color change of sodium nitroprusside solution upon standing and exposure to light has been attributed to the change of ferric to ferrous ion (57, 58).

When protected from light, aqueous solutions of sodium nitroprusside have been reported to be stable for as long as six months (11,59,60).

In aqueous solution the nitroprusside ion reacts with a wide variety of inorganic and organic substances to form usually highly colored reaction products (50, 52, 61-71).

Spectrophotometric measurements have most often been used to determine stability of sodium nitroprusside, with most emphasis on the increase in absorbance at 390-395 nm with degradation (11,49,50, 53,59,60). Burce has developed a stability

indicating method by complexing iron in any form other than nitroprusside with azide and measuring the resulting absorbance at 560 nm (72). Polarographic stability studies (11, 73) have indicated that the first two polarographic waves (Section 7.6) decrease in limiting current with degradation, but spectrophotometry is a much more sensitive method for detecting photodegradation (11).

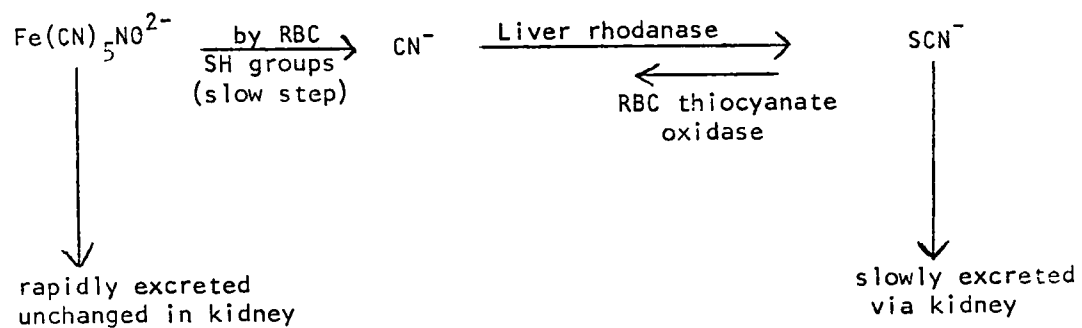
5. Drug Metabolic Products

When given intravenously, sodium nitroprusside rapidly lowers blood pressure by peripheral vasodilatation and reduction in peripheral resistance as a result of a direct action on the blood vessel walls, independent of autonomic innervation (74-78). Blood pressure can be maintained at any level depending on the rate of infusion (57,58). The hypotensive action is attributed to the nitroso (NO) group (57, 75-77, 79, 80) of the nitroprusside radical and is augmented in both dogs and humans by autonomic ganglion blocking agents (57,76).

The drug has an immediate effect, with desired blood pressure levels usually attained within 0.5 to 2 minutes. Upon discontinuation of the infusion, blood pressure rapidly rises to previous levels, usually within 1 to 10 minutes (81-84). This evanescence of the drug's effect is due to rapid destruction of the active nitroprusside radical which is slowly converted in the body to cyanide. This conversion is attributed to the interaction of the ferrous ion in nitroprusside with free sulfhydryl groups in erythrocytes (red blood cells) and other tissues (57,76,79,85,86). In vivo and in vitro studies have shown that nitroprusside liberates cyanide when contacted with liver (85), whole blood, washed erythrocytes, plasma, and urine (76,87-89). The release of cyanide is non-enzymatic (76,79,85,87), and its slow time course precludes the reaction from being the mechanism of action of nitroprusside (76,79). The cyanide is then converted by the hepatic enzyme rhodanase (transsulfurase) to thiocyanate (79,90). A small amount of the thiocyanate is oxidized back to cyanide by a thiocyanate oxidase present in erythrocytes (91,92) and perhaps also by a reversal of the rhodanase system (93). Boxer and Rickards (94) found these two compounds to be in dynamic equilibrium but that the equilibrium in vivo is far in the direction of thiocyanate. The half-life for excretion of thiocyanate is approximately seven days with normal renal function (95). A metabolic scheme (57) is presented in Figure 3.

FIGURE 3

Fate of Sodium Nitroprusside in the Body



RBC = red blood cells

 CN^- = cyanide SCN^- = thiocyanate

Oral administration of sodium nitroprusside for long periods does not significantly lower blood pressure; the effects are similar to those of thiocyanate given orally (76). Since thiocyanate accumulates in blood with prolonged infusion of sodium nitroprusside, thiocyanate or cyanide may be responsible for some later effects of the drug (57,76,77).

6. Toxicity

Sodium nitroprusside has few side effects, none of which usually requires discontinuance of therapy, provided that dosage is reasonable (57,58,79,81,82,96). Acute toxicity was initially thought due primarily to formation of cyanide, but subsequent studies (74) have indicated that the immediate toxicity of the drug is probably due to severe hypotension, caused by excessively high rates of infusion (57,58,79). Johnson (74) estimated the ratio between depressor and toxic dosages as 1:10.

Caution should be exercised in treatment with sodium nitroprusside since its immediate metabolic products are thiocyanate and cyanide (Section 5). Prolonged treatment may result in elevated serum thiocyanate levels, especially if renal function is impaired (57,76,97). Toxic symptoms of excessive elevation of thiocyanate in the blood include fatigue, nausea, weakness and loss of appetite (58,76). In a patient with severe renal insufficiency, long-term sodium nitroprusside administration resulted in hypothyroidism, caused by thiocyanate inhibition of the uptake and binding of iodine by the thyroid (97). Although significant levels of thiocyanate have appeared in blood during chronic oral administration of nitroprusside (76), elevated levels have not been observed with its short-term use (81) or during prolonged, intravenous use (98) in patients with normal kidney function.

A small amount of thiocyanate is oxidized back to cyanide in the body (Section 5). Elevated blood cyanide levels in vivo have been reported following sodium nitroprusside administration (87,88,92,94), but in the vast majority of cases the amounts have been small. Even with direct administration of therapeutic doses of thiocyanate, blood cyanide amounts were small and well below lethal concentrations (91,92). Vesey et al. (88) found a significant rise in plasma cyanide levels after sodium nitroprusside infusion and a simultaneous decrease in plasma vitamin B₁₂ (99), although there were no adverse effects on the

patients. Since the liver serves as the main regulatory system of cyanide detoxification (Section 5), sodium nitroprusside should be used with caution in patients with impaired liver function (57,88,97,100).

Sodium nitroprusside infusion to baboons was studied and, on a weight correction basis, it has been reported that the smallest toxic dose in the baboon given over 1-1/2 - 2 hours is equivalent to 320 mg/hour in man, and the mean toxic dose equivalent to 518 mg/hour (101). Intravenous LD₅₀ has been determined to be 8.4 ± 0.3 mg/kg in mice, 11.2 ± 1.1 mg/kg in rats, 2.8 ± 1.1 mg/kg in rabbits, and approximately 5 mg/kg in dogs (102). LD₅₀ in mice has been determined to be 48 ± 2.9 mg/kg orally and greater than 2000 mg/kg topically (103).

7. Methods of Analysis

7.1 Elemental Analysis

An elemental analysis of a standard sample of sodium nitroprusside (as the dihydrate) is presented in Table IV. Water was determined by Karl Fisher titration (104).

TABLE IV

Elemental Analysis of Sodium Nitroprusside

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
C	20.14	20.12
H	1.34	1.40
N	28.21	29.68
Na	15.44	14.98
Fe	18.74	18.72
<hr/>		
H ₂ O	12.09	12.03

7.2 Identification Tests

The visible absorption spectrum (Section 2.3) is specified by USP XIX as the identification test for sodium nitroprusside (105). The infrared spectrum (Section 2.1) may also be used for identification of

the drug.

For the dosage form, USP XIX specifies mixing sodium nitroprusside with ascorbic acid and dilute HCl, followed by dropwise addition of sodium hydroxide T.S. to produce a transient blue color (105). A number of other qualitative color reactions have been reported (106-110).

7.3 Thin-Layer Chromatographic Analysis

A number of TLC systems for the separation of sodium nitroprusside from its metabolites, thiocyanate (SCN^-) and cyanide (CN^-), are listed in Table V (111). Silica gel stationary phases were used in each, and nitroprusside was detected with 1% Na_2S in 0.5N NaOH, SCN^- with 0.1% FeCl_3 in 0.5N HCl, and CN^- with the method of O. Waschwik et al. (112). A good separation of the three substances is possible using the first system listed (solvent front 10 cm) followed by the second system (solvent front 14 cm), resulting in distances from starting point for CN^- , nitroprusside and SCN^- of 0, 45 and 99 mm, respectively (111).

TABLE V

Thin-Layer Chromatographic Systems for Sodium Nitroprusside

<u>Solvent</u>	<u>R_f Values</u>		
	<u>CN^-</u>	<u>Nitroprusside</u>	<u>SCN^-</u>
n-propanol:H ₂ O (10:2)	--	--	--
n-butanol:2N NH ₃ (1:1) (organic phase)	0	0.20	0.71
n-propanol:H ₂ O (10:1)	0	0.44	0.77
n-butanol:n-propanol: dibutylamine (45:45:10)	0	0.95	0.85

7.4 Spectrophotometric Analysis

Sodium nitroprusside may be analyzed spectrophotometrically by utilizing the molar absorptivity value ($\epsilon = 20.4$) at the maximum in the visible spectrum at 394 nm (11).

7.5 Colorimetric Analysis

Small amounts of nitroprusside have been determined colorimetrically as the isophorone complex by measuring absorbance at 495 nm in pH 10.2 buffer (113).

An indirect colorimetric method for sodium nitroprusside determination, consisting of precipitation with 1,10-phenanthroline, separation and measurement of the extinction coefficient of the filtrate, has been reported (114-115).

7.6 Polarographic Analysis

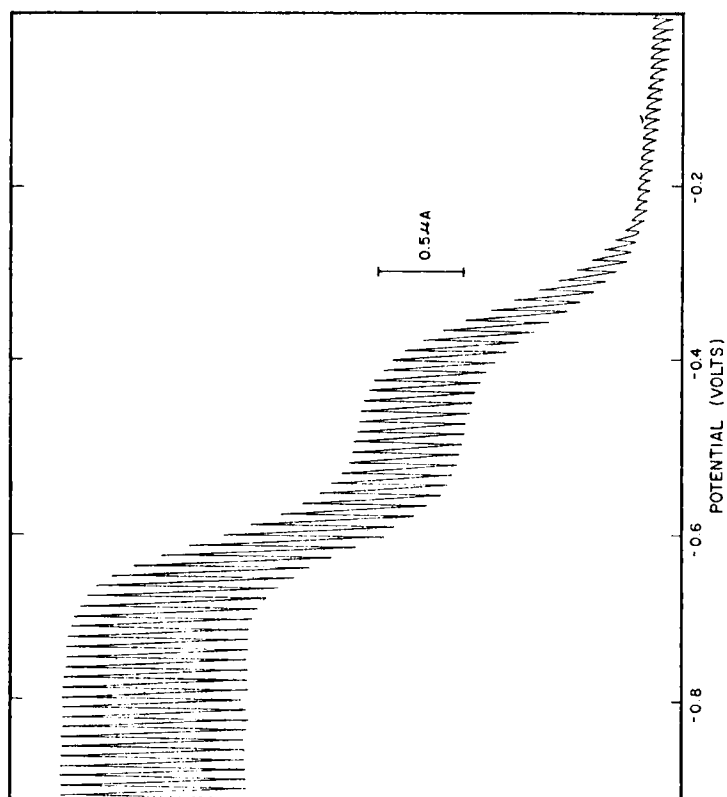
Sodium nitroprusside has been determined polarographically by a number of workers. At the dropping mercury electrode, three reduction waves were observed at -0.4, -0.6 and -1.2 volts vs. SCE. The first two waves were reported to involve one electron each as calculated from the n in the Ilkovic equation, are independent of the hydrogen ion concentration in the pH range 6 to 10, and are reversible, while the third wave is irreversible and the value of n is 2 (31,116,117). Zuman and Kabat (118,119) confirmed that the first two waves were one-electron reductions, and deduced that the third wave was a two-electron reduction, but considered all three waves to be irreversible. More recent studies (11,73,120) have reported the first two waves only. A typical polarogram of sodium nitroprusside, showing the first two waves, is shown in Figure 4 (120).

The current of the first polarographic reduction wave at about -0.33 volts vs. Ag/AgCl reference electrode in aqueous pH 7.2 buffer is used to assay the dosage form (50 mg dry-filled vial) (105,120). Photodegradation of sodium nitroprusside has also been determined by following the decrease in limiting current of the first two polarographic waves (11,73).

7.7 Coulometric Analysis

Coulometric studies of nitroprusside, using a mercury cathode and a silver anode, have indicated that the second and third reduction waves involve two and four faradays per mole of electrode reaction, respectively, while the products of reduction interfered with the determination of n for the first wave

FIGURE 4
Polarogram of Sodium Nitroprusside



(121,122). It has also been reported that controlled potential coulometric titration was not stoichiometric, probably due to competing background reactions (120).

7.8 Titrimetric Analysis

Sodium nitroprusside is assayed by dissolving the sample in water and titrating with 0.1N silver nitrate. The endpoint is determined potentiometrically, using a silver-silver chloride electrode system. Each ml of 0.1N silver nitrate is equivalent to 14.90 mg of $\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]\cdot 2\text{H}_2\text{O}$ (105). Alternatively, mercuric nitrate has been used as titrant, and polarized platinum electrodes and silicon-rubber based halide-selective membrane electrodes have been used as indicator electrodes (123). Titrimetric determination of nitroprusside with mercurous ion has been described by Tomicek and Kubik (124).

An indirect titrimetric method for nitroprusside, using a fluorescent endpoint, has been reported (125). After decomposition of nitroprusside with NaOH and $\text{Na}_2\text{Ni}(\text{CN})_4$ and filtration, the nickel is titrated with Na_2EDTA with bisglycinemethylenedichlorofluorescein as metallofluorochromic indicator.

7.9 Miscellaneous Methods of Analysis

Nitroprusside has been determined gravimetrically using diantipyrylphenylmethane (126), and by precipitation of nickel hydroxide in the reaction of nickel cyanide with alkaline nitroprusside (127). The latter method is more selective than the former, but cyanide, ferricyanide, and large amounts of ferrocyanide will interfere (113).

A microcrystal test, one in which the precipitate formed by the chemical reaction between a substance and a reagent is examined with a microscope, has been reported for the determination of sodium nitroprusside (128).

The variation of equivalent conductivities of aqueous solutions of sodium nitroprusside has been studied as a function of the ionic concentration (129).

8. Acknowledgments

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Literature surveyed through October, 1976.

SULPHAMERAZINE

Richard D. G. Woolfenden

CONTENTS

1. Description
 - 1.1. Name, Formula, Molecular Weight
 - 1.2. Appearance, Colour, Odour, Taste
2. Physical Properties
 - 2.1. Infra-red Spectrum
 - 2.2. Ultraviolet Spectrum
 - 2.3. Fluorescence and Phosphorescence Spectra
 - 2.4. Mass Spectrum
 - 2.5. N.M.R. Spectrum
 - 2.6. Melting Range
 - 2.7. Differential Thermal Analysis
 - 2.8. Thermal Gravimetric Analysis
 - 2.9. X-ray Diffraction
 - 2.10. Polymorphism
 - 2.11. Solubility
 - 2.11.1. In Aqueous Buffers and Urine
 - 2.11.2. In Solvents
 - 2.12. Dissociation Constant
 - 2.13. Partition Coefficients
3. Synthesis and Purification
 - 3.1. Chemical Synthesis
 - 3.2. Purification
4. Salts
 - 4.1. Organic Salts
 - 4.2. Metal Complex Salts
5. Chemical Stability
 - 5.1. Hydrolysis
 - 5.2. Pyrolysis
 - 5.3. Photolysis
6. Methods of Analysis
 - 6.1. Identification
 - 6.2. Elemental Analysis
 - 6.3. Titrimetric Assay Procedures
 - 6.3.1. Diazometric Titrimetry
 - 6.3.2. Non-Aqueous Titrimetry
 - 6.3.3. Bromometric Titrimetry
 - 6.3.4. Argentometric Titrimetry
 - 6.3.5. Complexometric Titrimetry
 - 6.3.6. Thermometric Titrimetry
 - 6.4. Spectrophotometric Assay Procedures
 - 6.4.1. Infra-red Spectroscopic Methods

- 6.4.2. Ultraviolet Spectroscopic Methods
 - 6.4.3. Colorimetric Methods
 - 6.5. Chromatographic Procedures
 - 6.5.1. High Performance Liquid Chromatography
 - 6.5.2. Gas Chromatography
 - 6.5.3. Thin Layer Chromatography
 - 6.5.4. Paper Chromatography
 - 6.5.5. Ion Exchange and Partition Chromatography
 - 6.5.6. Electrophoresis
 - 6.6. Electrochemical Methods
 - 6.6.1. Polarography
 - 6.6.2. Ion Selective Electrodes
 - 6.7. Bioassay
- 7. Estimation in Biological Fluids
- 8. Pharmacology
 - 8.1. Metabolism
 - 8.2. Absorption, Distribution, Excretion
 - 8.2.1. In Humans
 - 8.2.2. In Animals
 - 8.3. Toxicity
 - 8.3.1. Acute Toxicity
 - 8.3.2. Chronic Toxicity
 - 8.3.3. Clinical Toxicity
- 9. Protein Binding
- 10. Pharmacodynamics
- 11. Acknowledgements
- References

1. Description

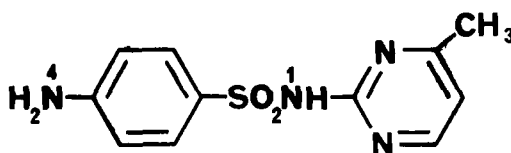
1.1. Name, Formula, Molecular Weight

Generic names¹ - Sulphamerazine;
Methylpyrimal; Sulphamethyldiazine.

Nomenclature - The following nomenclature is used in Chemical Abstracts:
N¹-(4-methyl-2-pyrimidinyl)sulphanilamide; 4-amino-N-(4-methyl-2-pyrimidinyl) benzenesulphonamide.

Structure

Chemical Abstracts Registry No.
(127-79-7)



C₁₁H₁₂N₄O₂S

Mol.wt 264.30.

1.2. Appearance, Colour, Odour, Taste²

White or faintly yellowish white crystalline powder which is odourless but has a slightly bitter taste. It is stable in air but slowly darkens on exposure to light.

2. Physical Properties

2.1. Infra-red Spectrum

The infra-red spectrum of sulphamerazine (Squibb sample PO83425) was recorded³ in KBr and is shown in Figure 1. Assignments for the more important absorption bands are listed in Table 1.^{4,5}

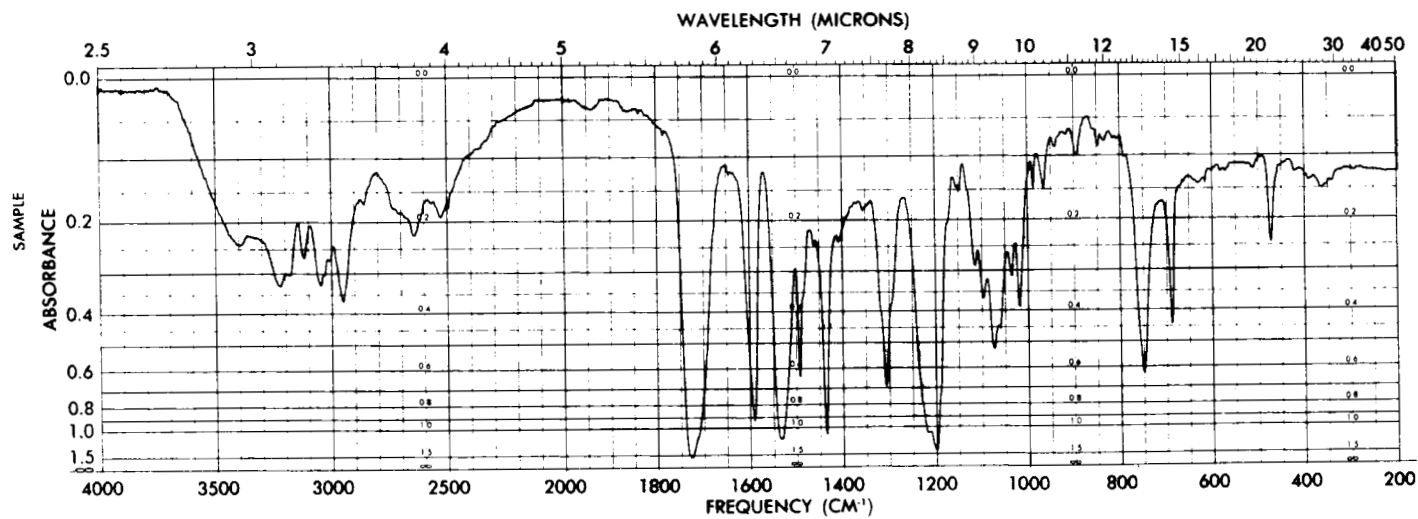


Fig.1 Infra spectrum of sulphamerazine (KBr pellet)

TABLE 1Infrared assignments for Sulphamerazine

<u>Frequency (cm⁻¹)</u>	<u>Assignment</u>
3490	NH asymmetric stretching.
3380	NH symmetric stretching.
2960	CH ₃ asymmetric stretching.
2870	CH ₃ symmetric stretching.
1630	NH ₂ scissoring.
1600)	C = C stretching, skeletal
1570)	vibrations of aromatic
1500)	ring.
1325	SO ₂ asymmetric stretch
	overlapping C-N stretch-
	ing vibration.
1160	SO ₂ symmetric stretching.
1092	Aromatic CH in plane
	bending.
890	S-N stretching.
835	C-H out of plane deforma-
	tion.

2.2. Ultraviolet Spectrum

The ultraviolet spectrum of sulphamerazine in 0.1M hydrochloric acid solution exhibited³ absorption maxima at 243 nm and at 307 nm (Figure 2). In 0.1M sodium hydroxide solution sulphamerazine behaves as the sodium salt exhibiting one main peak with two maxima appearing at 243 nm and 257 nm as shown in Figure 3. The hypsochromic shift of the 307 nm maximum to 257 nm in alkaline solution is due to ionization of the sulphonamide fraction of the molecule. The ultraviolet spectrum of sulphamerazine has been recorded in water⁶ (maxima at 243 and 257 nm) and 95% ethanol⁶ (maximum at 271 nm). The $E_{1\text{cm}}^{1\%}$ values evaluated for the aforementioned systems are given in Table 2.

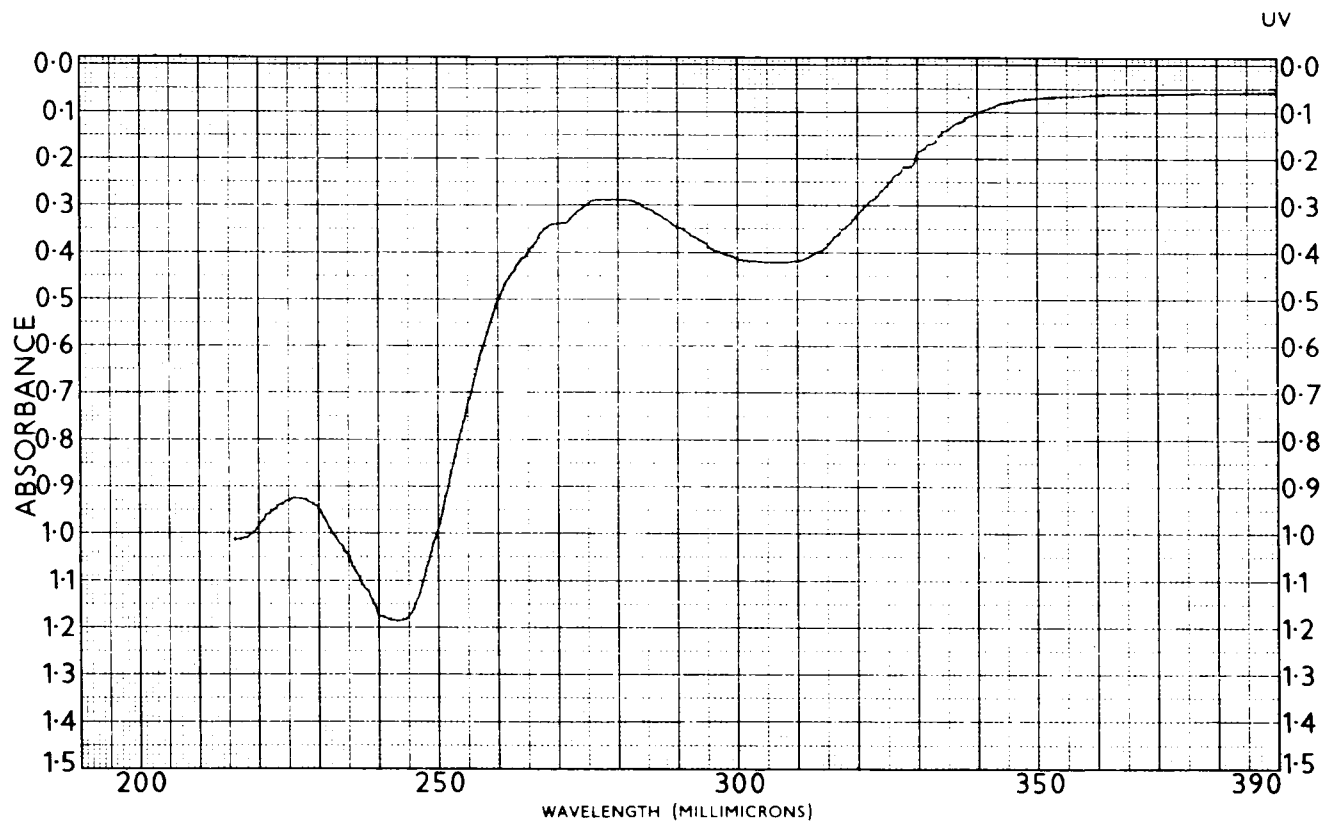


Fig. 2 Ultraviolet spectrum of sulphamerazine (0.1N HCl)

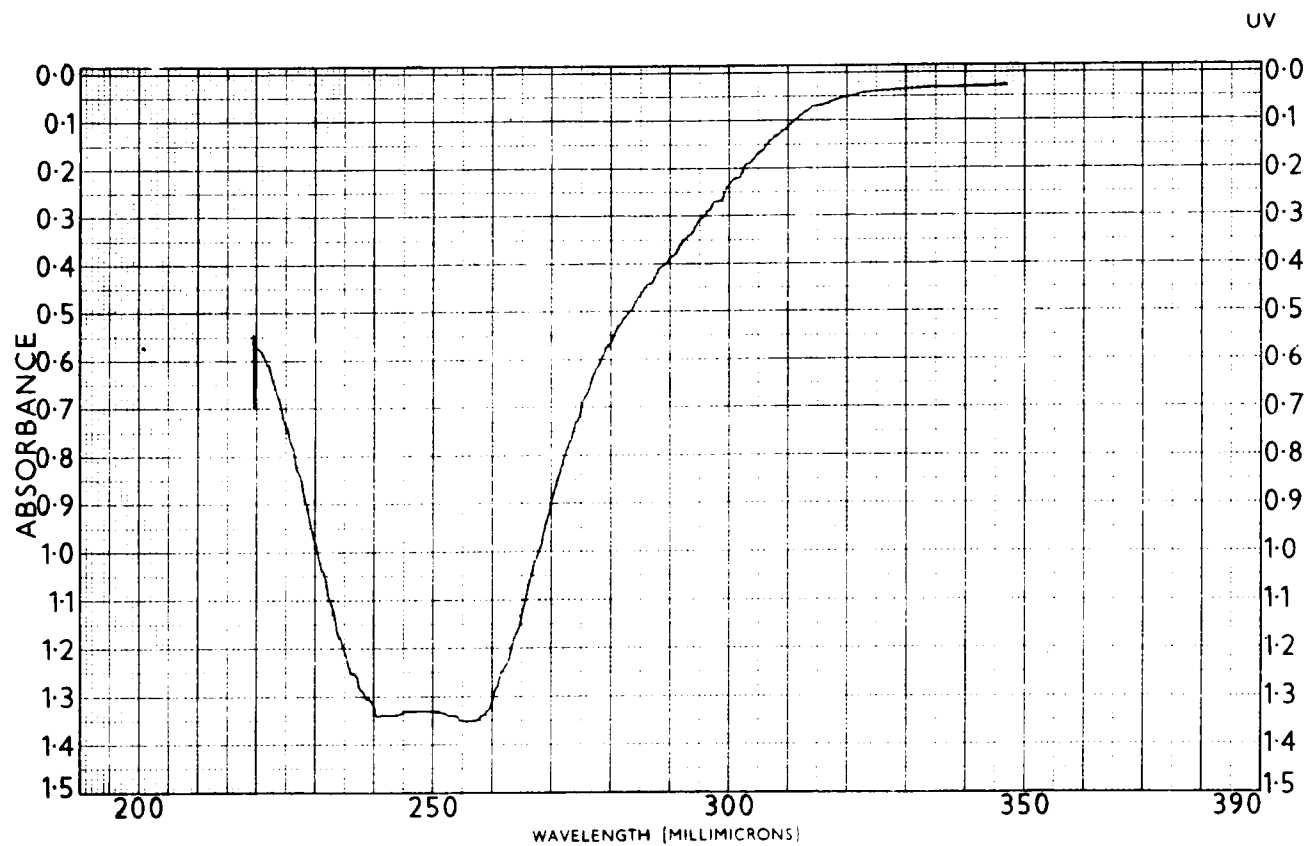


Fig.3 Ultraviolet spectrum of sulphamerazine (0.1N NaOH)

TABLE 2
 $E_{1\text{cm}}^{1\%}$ values for sulphamerazine in
various solvent systems

<u>Solvent</u>	<u>Band(nm)</u>	<u>$E_{1\text{cm}}^{1\%}$</u>	<u>Reference</u>
0.1M HCl aqueous	243	579	7
		625	3
	307	200	3
0.1M NaOH aqueous	243	896	3
	257	883	3
Water	243	875	6
	257	822	6
95% Ethanol	271	835	6

2.3. Fluorescence and Phosphorescence

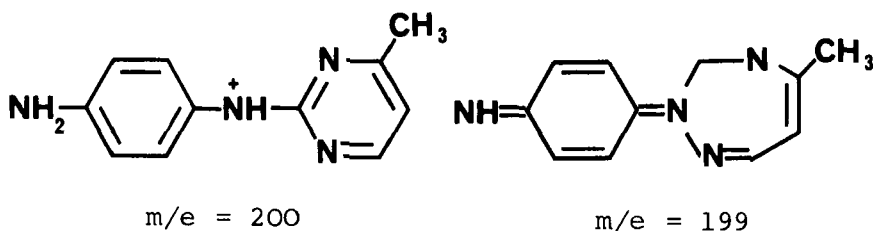
N^1 - Substituted sulphonamides containing a π -electron deficient heterocyclic ring system are generally weakly or non-fluorescent. Sulphamerazine is such a sulphonamide and its lack of fluorescence has been demonstrated by Gifford and co-workers⁸. The presence of the heterocyclic ring at the N^1 -position produced a marked quenching of fluorescence over the pH range studied. This observation was a general feature of N^1 -substituted heterocyclic sulphanilamides and it was considered that these compounds preferentially absorbed light via an $n \rightarrow \pi^*$ transition⁹ which is known to detract from fluorescence.

Sulphamerazine has been shown to exhibit a phosphorescence spectrum originating from a transition in the lowest excited triplet level in the aromatic nucleus. Gifford and co-workers⁸ produced the phosphorescence and emission spectrum of sulphamerazine at 77°K using a Baird-Atomic SF 100-E spectrofluorimeter fitted with a phosphoroscope attachment, the excitation spectrum showing a maximum at 310nm (λ_e) and the emission spectrum a maximum at 412nm(λ_p). The delayed luminescence lifetime (τ) was 0.8 seconds.

2.4. Mass Spectrum

The mass spectrum of sulphamerazine shown in Figure 4 was obtained on an AEI-MS 902 mass spectrometer by direct sample introduction into the source at 90°C¹⁰. The fragmentation patterns which can be assigned to the more important ions are shown in scheme I^{3,10,11}.

Campan and co-workers¹¹ have studied the mass spectra of several sulphapyrimidines and showed that preferential fragmentation occurred to eliminate SO₂. The fragmentation patterns were attributed to localization of the charges on the heteroatoms. The workers considered the peaks at m/e = 200 and m/e = 199 as extremely important corresponding to the removal of SO₂ and SO₂H to give the following ions:



2.5. N.M.R. Spectrum

Puar and Funke¹⁰ have recorded the 60 MHz N.M.R. spectrum of sulphamerazine in dimethyl sulphoxide - d₆ containing T.M.S. as internal standard (Figure 5). The structural data is presented in Table 3.

The natural abundance ¹³C magnetic resonance spectrum of sulphamerazine has been compared with a series of other sulphonamides by Chang and Floss¹³. The spectra were determined at 25.15 MHz using the pulse Fourier transform technique. Chemical shifts were assigned with the aid of off-resonance and selective proton decoupling techniques as well as by long-range ¹³C proton coupling patterns.

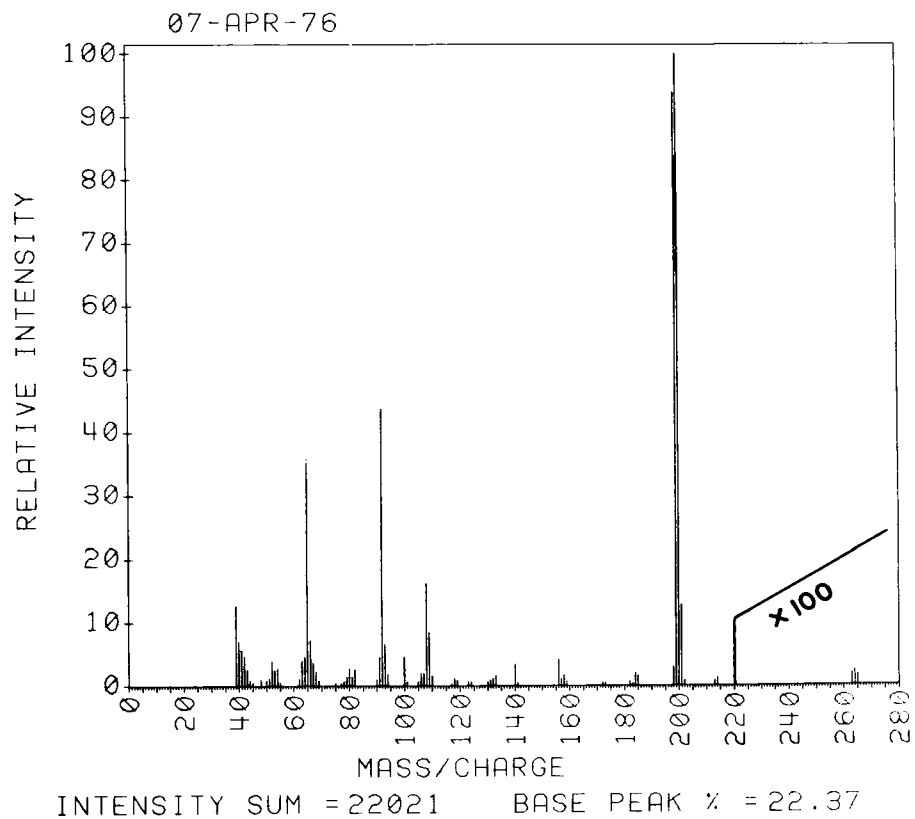
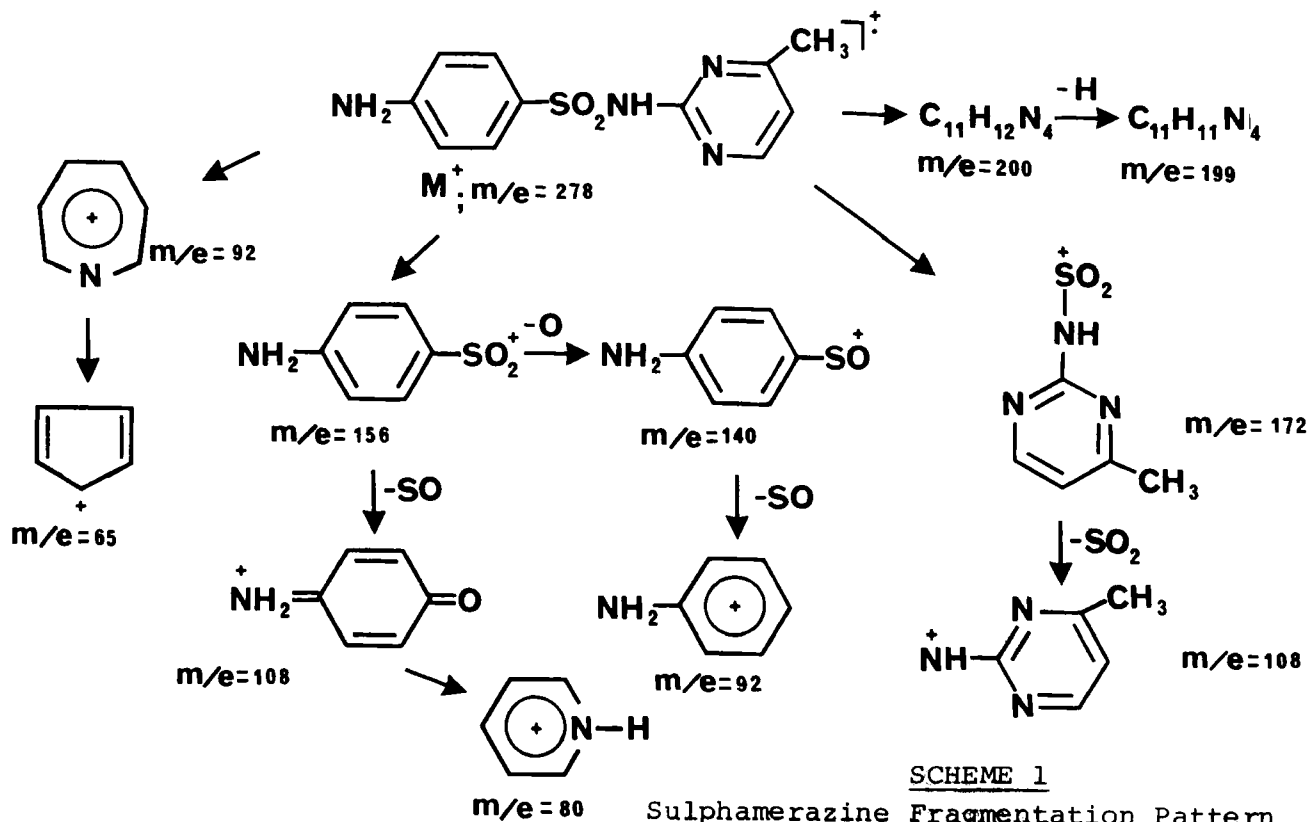


Fig. 4 Mass spectrum of sulphamerazine



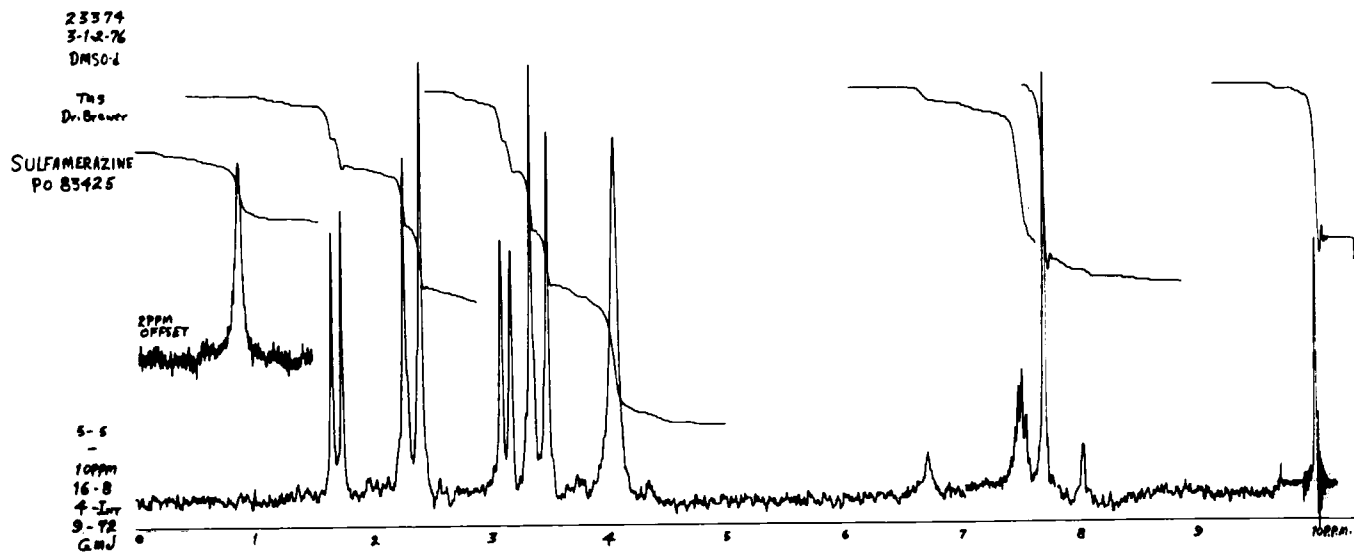


Fig. 5 N.M.R. spectrum of sulphamerazine (DMSO-d₆)

TABLE 3

NMR Spectral Assignments of Sulphamerazine^{10,11}

<u>Proton Assignment</u>	<u>Chemical shift, δ (ppm)</u>	<u>J (Hz)</u>
2H } p-substituted	6.57d	9.0
2H } benzene ring protons	7.70d	9.0
1H } Vicinal	6.86d	5.0
1H } heterocyclic protons	8.30d	5.0
3H CH ₃	2.29s	
2H NH ₃	5.95b,s	
1H NH ₂	11.12b,s	

s = singlet; d = doublet; b = broad.

2.6. Melting Range

The melting range quoted in the U.S.P. XIX is 234-239°C. A melting point of 234°C was obtained for a U.S.P. grade sample of sulphamerazine using D.T.A.³.

2.7. Differential Thermal Analysis

Using a Stanton Redcroft Thermal Analyser Model 671 at a heating rate of 20°C min⁻¹, it was found that U.S.P. grade sulphamerazine gave a sharp melting endotherm at 234°C³ (Figure 6). This was rapidly followed by decomposition. The heat of fusion (ΔH_f) evaluated by Yang and Guillory¹⁴ was 8.68 k.cal.mol⁻¹, at a fusion temperature of 236°C whereas Sunwoo and Eisen¹⁵ quote a value of 7.54 k.cal.mol⁻¹, at a fusion temperature of 242°C. Yang and Guillory also quoted an entropy of fusion of 17.1 e.u. for sulphamerazine.

2.8. Thermal Gravimetric Analysis

The thermogravimetry of sulphamerazine has been studied by Cook and Hildebrand¹⁶. Sulphamerazine exhibited no weight loss up to a temperature of 260°C, but between 260°C and 396°C a rapid weight loss occurred followed

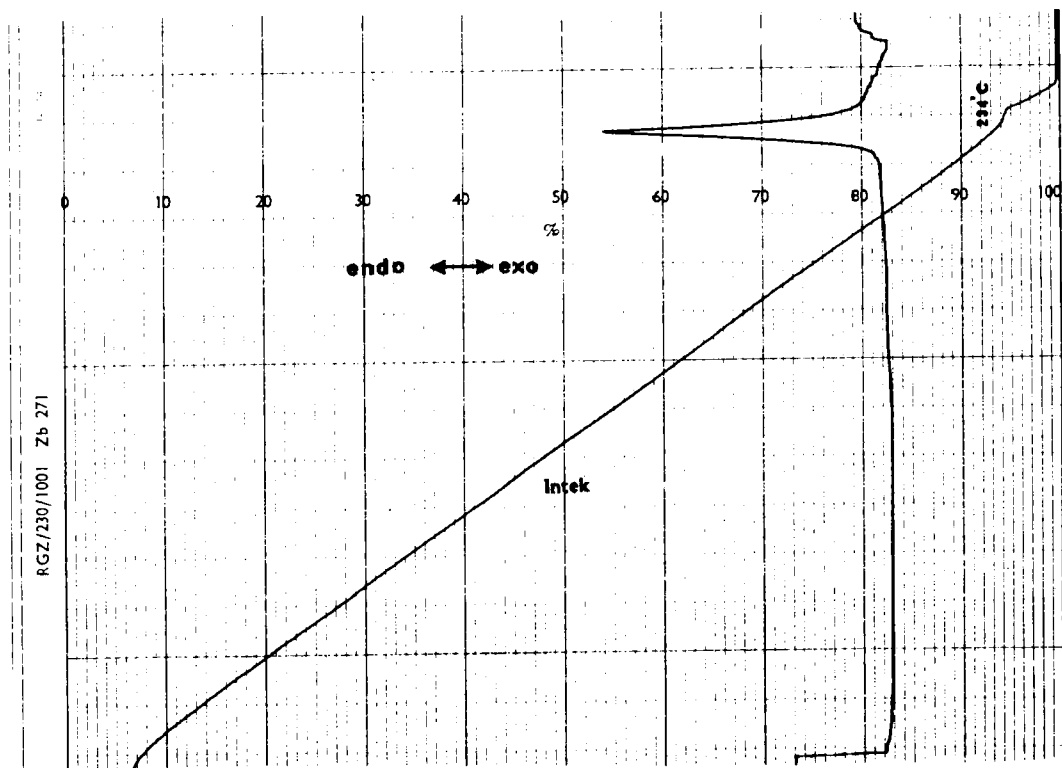


Fig 6. DTA of sulphamerazine

by a less rapid loss between 526°C and 690°C. The TGA curve, therefore, exhibited plateaus at temperature ranges 396-526°C. No residue remained at the end of the heating period. Although no attempt was made to identify the gaseous pyrolysis products Cook and Hildebrand hypothesised that sulphur dioxide would probably split out from the sulphamerazine molecule in a similar manner to sulphones and alkylsulphonyl chlorides.

2.9. X-ray Diffraction

Ochs¹⁷ has recorded the X-ray powder diffraction pattern for a sample of sulphamerazine (see Figure 7 and Table 4). Yang and Guillory¹⁴ and Lennox¹⁸ have also reported X-ray powder diffraction data for sulphamerazine.

TABLE 4
X-Ray Powder Diffraction Data of Sulpha-
merazine (PO83425)

<u>Interplanar Distances</u>	<u>Relative Intensities</u>
$d, \text{\AA}^*$	I/I_0
10.72	0.117
7.65	0.130
7.03	0.949
6.76	0.315
6.35	0.199
6.02	0.885
5.46	0.636
5.14	0.760
4.72	0.207
4.37	0.971
4.11	0.545
3.95	0.432
3.89	0.322
3.30	0.257
3.74	0.307
3.67	1.000
3.53	0.324
3.27	0.207
3.22	0.286
3.05	0.142
2.94	0.133
2.90	0.397
2.76	0.456
2.38	0.278

$$*\text{Interplanar distance } d = \frac{n \lambda}{2 \sin \theta}$$

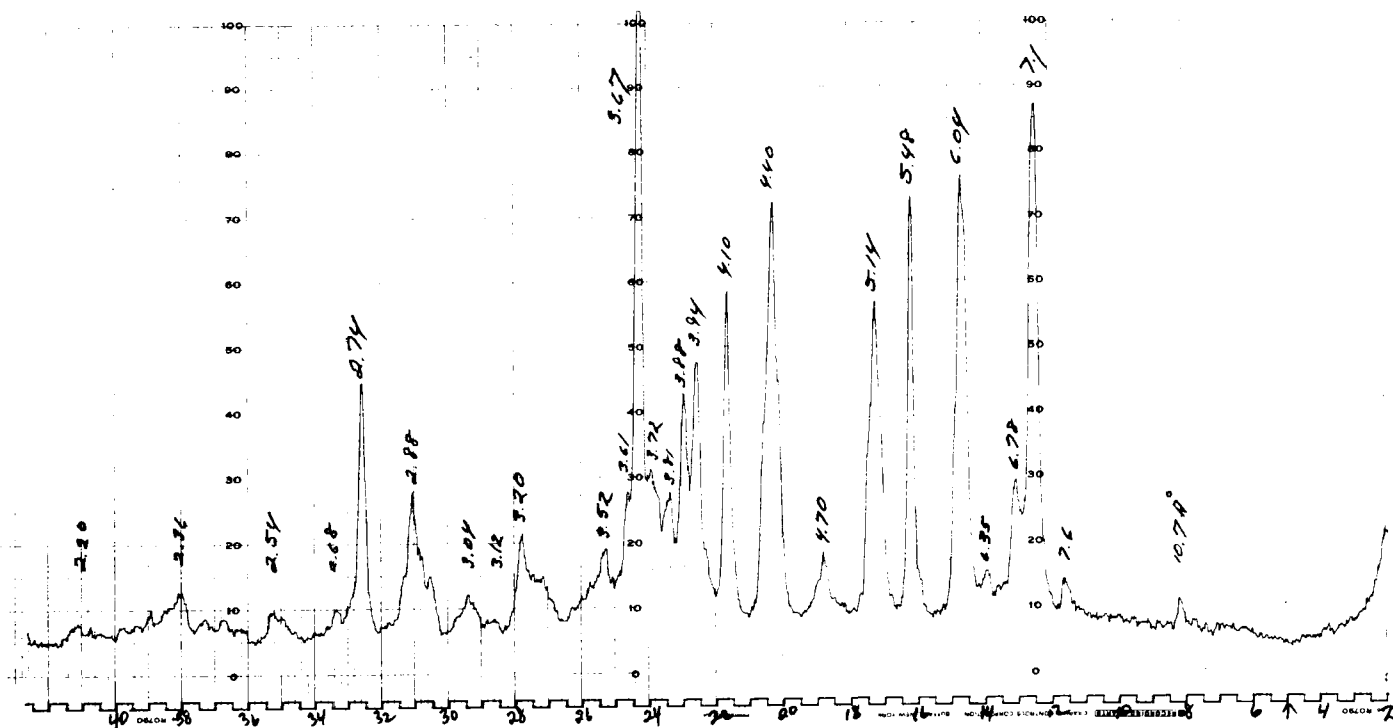


Fig. 7 X-Ray powder diffraction pattern of sulphamerazine

2.10. Polymorphism

During extensive studies on polymorphism in sulphonamides using X-ray diffraction, infrared and D.T.A. techniques Yang and Guillory¹⁴ found that sulphamerazine was among those sulphonamides in which polymorphism could not be detected.

2.11. Solubility

2.11.1. In Aqueous Buffers and Urine

The solubility of sulphamerazine in aqueous media is important in clinical practise and therefore, it has mainly been determined in aqueous buffers and urine in the approximate pH range of 6-8 at 37°C. Typical values are given in Table 5 along with those of the N⁴-acetyl derivative.

TABLE 5

The Solubility of Sulphamerazine and its N⁴-acetyl derivative in aqueous phosphate buffer and urine at 37°C.

<u>Medium</u>	<u>Solubility</u> <u>mg./ml.</u>		<u>Reference</u>
	<u>Sulpha-</u> <u>merazine</u>	<u>N⁴-Acetyl-</u> <u>sulphamera-</u> <u>zine</u>	
M/30 Phosphate buffer, pH 6.1	40	53	19
Urine, pH 5.9	37	76	19, 20
Urine, pH 6.9	66	175	19, 20
Urine, pH 7.9	310	650	19, 20

2.11.2. In Solvents

The approximate solubilities of sulphamerazine in some solvents are given in Table 6.

TABLE 6
Sulphamerazine solubilities in
some solvents

<u>Solvent</u>	<u>Solubility</u> <u>mg./ml.</u>	<u>Reference</u>
Water, 20°C	16	6
Water, 37°C	30	6
Water, 100°C	330	6
1.5N Aqueous	290	21
NaOH, 22°C		
Ethanol, 22°C	330	6
Isopropanol, 22°C	174	22

2.12. Dissociation Constant

The dissociation of the primary aromatic amine function of some sulphonamides has been studied by Salvesen and Schroder-Nielson²³ using spectrophotometric and potentiometric methods. In 0.5M aqueous sodium chloride solution at 24°C the pK_{a1} value representing the primary amine dissociation of sulphamerazine was given as 2.29. Koizumi and co-workers²⁴ quoted a pK_{a1} value of 2.26.

Krebs and Speakman²⁵ determined the pK_{a2} of a number of sulphonamides from solubility data using the following relationship.

$$S = S^0 (1 + 10^{pH-pK_a})$$

where S is the solubility of the compound at a particular pH and S^0 is the solubility of the unionised compound. These workers obtained a pK_{a2} value of 6.95 ($S^0 = 41$ mg./100ml.) for the dissociation of the sulphonamide group of sulphamerazine in a solution of ionic strength 0.1 at 38°C. Using the same principle Sjogren and Ortenblad²⁰ obtained a pK_{a2} value of 7.05. Both these reports assumed that the sulphonamides behaved as monobasic acids. The authenticity of these pK_{a2} values has been confirmed by Willi and Meier²⁶, who using a potentiometric method, obtained a value of 6.84 at 20°C at an ionic strength of 0.1.

2.13 Partition Coefficients

During their studies on some pharmacokinetic aspects of certain sulphonamides Koizumi and co-workers²⁴ generated partition coefficient data at 37°C between an aqueous phase containing unionised drug and the solvents carbon tetrachloride, benzene, chloroform and isoamyl acetate. Suzuki and co-workers²⁷ also generated similar data using isoamyl alcohol as the organic phase. The results for sulphamerazine are given in Table 7.

TABLE 7
Partition Coefficients for sulphamerazine
at 37°C^{24,27}

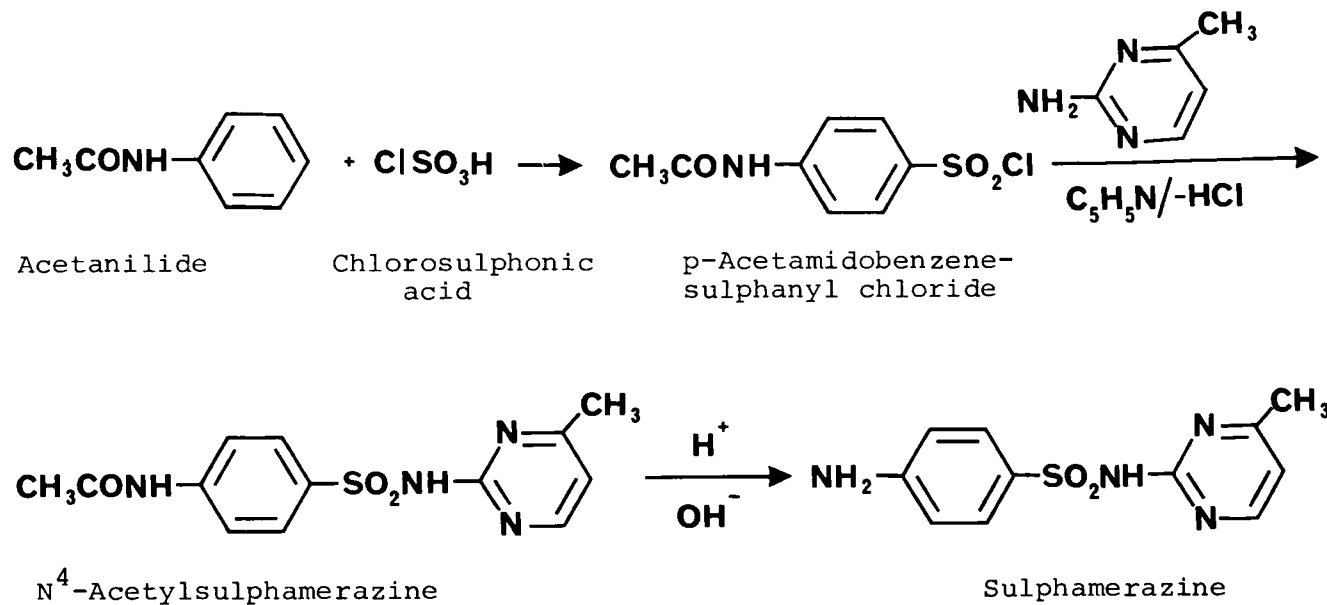
<u>Organic Phase</u>	<u>Partition Coefficient</u>
CCl ₄	0.022
C ₆ H ₆	0.202
CHCl ₃	2.4
Isoamyl acetate	2.1
Isoamyl alcohol	2.1

3. Synthesis and Purification

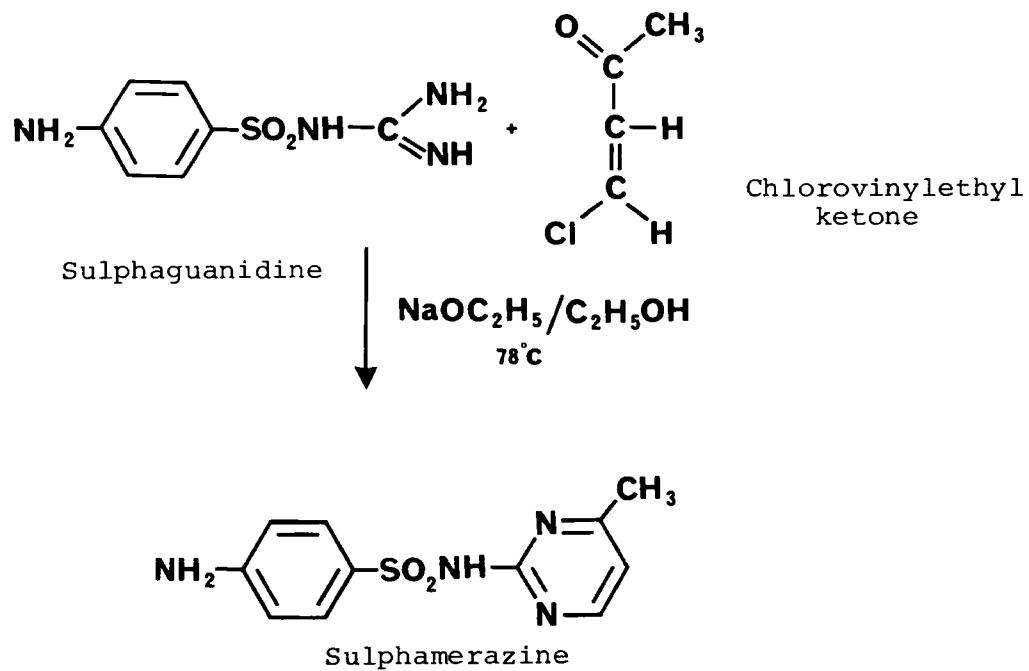
3.1. Chemical Synthesis

Two primary synthetic routes have been used to prepare sulphamerazine, these being via the reaction between 2-amino-4-methylpyrimidine with certain derivatives of benzenesulphonyl chloride and also by a condensation process between sulphaguanidine and certain ring forming compounds.

Roblin and co-workers²⁸ first synthesized sulphamerazine by the action of p-acetamidobenzene-sulphonyl chloride on 2-amino-4-methylpyrimidine in a weakly basic solvent such as pyridine to give the N⁴-acetyl derivative of sulphamerazine. Hydrolytic deacetylation of this intermediate was achieved under either acidic or basic conditions. Using acetanilide as the starting material the various steps involved in the synthesis are shown in Scheme 2. The p-nitro derivative of benzenesulphonyl chloride could also



SCHEME 2 - Synthesis of Sulphamerazine



SCHEME 3 - Synthesis of Sulphamerazine

be used, the final stage of the synthesis requiring a catalytic reduction of the nitro group to give the final product.

In the second major method a number of ring forming compounds were condensed with sulphaguanidine to produce sulphamerazine. Typically sulphaguanidine has been condensed with chlorovinylmethyl ketone in alkaline medium²⁹ as illustrated in Scheme 3. In this case the condensation mechanism involved the removal of a molecule of water and a molecule of hydrochloric acid to give the final product. Other ring forming compounds which have been used include acetoacetaldehyde acetals³⁰, acetaldehyde methyl acetals³¹, and dialkylaminobutenynes³².

3.2. Purification

Crude sulphamerazine is usually purified via its sodium salt. In one method³³ the pH of the medium was adjusted to 10.5 by the addition of calcium hydroxide. The solution was boiled and sodium dithionite added. Decolorization was then achieved using activated charcoal. On cooling to room temperature the solution was acidified with acetic acid and the precipitated sulphamerazine isolated by filtration. If required this product could be recrystallised from aqueous alcohol or benzene. A number of variations on this theme have been described^{29,34,35}.

4. Salts

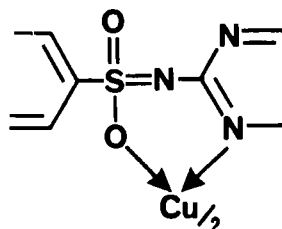
4.1. Organic Salts

Barry and Puetzer³⁶ prepared the cetyl-methylammonium sulphamerazine dihydrate salt which was found to have a melting point of 126°C. Schonhofer³⁷ prepared the diethylaminoethanol salt of sulphamerazine which was found to give a 30% aqueous solution of pH 9.2-9.5. Winnek³⁸ prepared aqueous solutions of streptomycin sulphate and barium or calcium salts of certain sulphonamides in varying proportions to give salts of streptomycin containing 1,2 or 3 moles of sulphonamide.

The streptomycin disulphamerazine salt was found to have a water solubility of about 10%.

4.2. Metal Complex Salts

Various complex salts of sulphonamides with multivalent metals have been prepared. Complex salts of cobalt, sulphamerazine³⁹ and ethylenediamine were prepared by Erdos³⁹ at a temperature of less than 5°C for 24 hours followed by precipitation with 100 ml. ethanol. Shakh⁴⁰ prepared the cobalt, nickel, copper and zinc complexes of sulphamerazine and found them to be insoluble in water, alcohol, ether, chloroform, acetone and benzene. These complexes were found to be soluble in acid solution but were decomposed by 10% sodium hydroxide or ammonia. The molar ratio of sulphamerazine to metal was 2:1. Lee has studied in depth the formation of copper complexes of the sulphonamides, dealing with their preparation from copper acetate⁴¹, their sensitivity to micro-organisms⁴², the determination of their stability constants⁴³, and their structure assignments⁴⁴. The copper complex of sulphamerazine was prepared by treating an alcoholic solution of the sulphonamide with an aqueous solution of cupric acetate at pH 7-9. The complex was isolated as grey needles, was less sensitive to micro-organisms than sulphamerazine, and had a stability constant of 9.68 at 25°C. The structure of the complex was determined⁴⁴ by infrared spectroscopy which exhibited a shift in the S = O absorption band from 7.62μ for sulphamerazine to 7.79μ in the copper complex. From the infrared data it was deduced that the copper chelated between the S-O group of the sulphonamides and a heterocyclic nitrogen atom as follows



5. Chemical Stability

5.1. Hydrolysis

The kinetics of the acid catalysed hydrolysis of some sulphanilamidopyrimidines has been studied by Zajac⁴⁵. The hydrolysis rate was found to follow first order kinetics in each case, the rate being dependent on the hydrogen ion concentration. The results of the study also showed that the substitution of methyl or methoxy groups within the pyrimidine nucleus increased the hydrolysis rate. Thus the half life of the sulphamerazine hydrolytic process at 60°C (333°K) was found to be 67.9 hours compared to 94.7 hours for sulphadiazine the parent sulphanilamidopyrimidine.

Auterhoff and Schmidt⁴⁶ also studied the hydrolysis of certain sulphanilamidopyrimidines. Using TLC combined with elemental, analytical and spectroscopic techniques these investigations identified sulphanilic acid, sulphanilamide, 2-amino-4-methylpyrimidine and 2-hydroxy-4-methylpyrimidine as the decomposition products of sulphamerazine.

5.2. Pyrolysis

The pyrolytic decomposition of sulphanilamidopyrimidines was also studied by Auterhoff and Schmidt⁴⁶. The compounds were placed into test tubes and heated in an oil bath to between 230 and 280°C. Yellowish white sublimes appeared in the upper part of the test tubes which were subsequently examined by TLC on Merck Kieselgel F254 using n-butanol, acetic acid, water (80,20,20) as solvent system. Sulphamerazine (R_f 0.59) was found to decompose to 2-amino-4-methylpyrimidine (R_f 0.48) in 92% yield.

5.3. Photolysis

Naito and Mizoguchi⁴⁷ studied the photolytic decomposition of certain sulpha drugs and their benzoyl derivatives in aqueous alkaline solution using a sterilization lamp. An ultraviolet spectrophotometric assay method

showed that about 50% of sulphamerazine was decomposed over a period of 8 hours whereas the benzoyl derivative was completely stable. The same samples stored in the dark exhibited no decomposition.

6. Methods of Analysis

6.1. Identification

Two identity tests are given in the U.S.P.XIX, one being an infrared absorption test and the other a microchemical test. In the latter method a sample of sulphamerazine is suspended in water and the suspension made alkaline with sodium hydroxide. On the addition of cupric sulphate solution an olive green precipitate is formed which turns dark grey on standing. This test has been successfully used^{48,49} to detect sulphamerazine in the presence of other sulphonamides. Turczan and Medwick¹² have included sulphamerazine in a classification scheme for the identification of sulphonamides by N.M.R.spectroscopy.

6.2. Elemental Analysis

The elemental composition of sulphamerazine (Squibb batch PO 83425) was obtained by Young⁵⁰ with the following results:-

<u>Element</u>	<u>% Theory</u>	<u>% Found</u>
Carbon	49.98	50.08
Hydrogen	4.58	4.55
Nitrogen	21.20	21.31
Sulphur	12.13	12.12
Oxygen	12.11	-

6.3. Titrimetric Assay Procedures

6.3.1. Diazometric Titrimetry

Sulphamerazine may be titrated in strongly acid solution with a standard solution of sodium nitrite, the end-point being detected with an external or internal indicator, or by an electrometric procedure. The diazometric technique is

the official method of the U.S.P. XIX for sulphamerazine.

El-Sebai and co-workers⁵¹ have evaluated the sodium salt of 4-(benzylamino)azo-benzene-4'-sulphonate as an internal indicator for sulphonamide titrations and claim that it provides a rapid, sharp and easily detected colour change which is stable for 30 minutes. More accurate results were obtained than with an external indicator such as starch iodide paper. Other internal indicators which have been successfully used are dicyanobis (1,10-phenanthroline) iron (II)⁵² and tropaeolin OO with methylene blue as contrast medium⁵³.

The diazometric method has been used for the determination of sulphamerazine in tablet dosage forms without interference from excipients such as starch, lactose, calcium carbonate, sodium bicarbonate, magnesium stearate, stearic acid, gelatin, gum acacia, and talc⁵⁴.

6.3.2. Non-Aqueous Titrimetry

The powerful electron withdrawing sulphonyl group in sulphonamides renders the amide hydrogen atom acidic so that these drugs can be conveniently titrated with a suitable base in a non-aqueous medium. Faber⁵⁵ titrated sulphamerazine in pyridine solution using sodium methoxide dissolved in a mixture of benzene and methanol (3:1) as titrant and thymol blue in methanol as indicator. Acidic tablet excipients were naturally found to interfere. Sulphamerazine has also been determined⁵⁶ in tetramethylurea with tetrabutylammonium hydroxide (0.1M) as titrant, the end-point being determined using either potentiometry or a thymol blue indicator.

More recently Davis and co-workers⁵⁷ evaluated 3-methyl-2-oxazolidone as a suitable solvent for the non-aqueous titration of sulphonamides on the basis that its high dielectric constant and wide liquid range contributed to its outstanding solvent

properties. Tetrabutylammonium hydroxide was used as titrant and potentiometry as end-point detection.

6.3.3. Bromometric Titrimetry

The bromometric methods of Wojahn⁵⁸ and Conway⁵⁹ are well established and have been applied, with excellent results by De Reeder⁶⁰ to the assay of sulphamerazine in mixtures with other sulphonamides.

Recently, however, some attention has been paid to the improvement of the detection of end-point in the bromometric method. Ejima and co-workers⁶¹ titrated a number of sulphonamides, including sulphamerazine, by a coulometric method involving bromination with electrolytically generated bromine in an aqueous solution of hydrochloric acid and potassium bromide. The end-point was detected potentiometrically. A coulometric method was also adopted by Ebel and co-workers⁶² in which excess of electrolytically generated bromine was titrated with cuprous ions to a potentiometric end-point.

A spectrophotometric titration with bromide-bromate solution has been developed⁶³, the drug being dissolved in a mixture of concentrated hydrochloric acid-acetic acid(2:8). Quantitative recoveries for sulphamerazine were reported as 98.43 ± 0.58% with bromination time of 5 minutes.

6.3.4. Argentometric Titrimetry

The principle of the argentometric method is that some sulphonamides form insoluble silver salts. The sulphonamides are precipitated by the addition of excess standard silver nitrate solution, the precipitate removed by filtration, and the excess silver nitrate titrated with standard ammonium thiocyanate using ferric alum as the indicator. De Reeder⁶⁴ successfully applied the above method to the determination of a mixture of sulphamerazine, sulphadiazine and sulphamethazine.

6.3.5. Complexometric Titrimetry

Abdine and Sayed⁶⁵ developed a complexometric assay for sulphamerazine. The sample was dissolved in alkaline solution and precipitated with excess copper sulphate solution in pH 6 borate buffer. The excess copper was then determined by titration with the disodium salt of E.D.T.A. using 1-(2-pyridylazo)-2-naphthol as indicator.

Sulphamerazine has also been estimated⁶⁶ in combined sulpha drugs by precipitation with excess copper acetate followed by the determination of the residual copper by complexing with E.D.T.A. The selective precipitation and complexometric assay of mixtures of sulphamerazine, sulphathiazole, and sulphadiazine were also discussed.

6.3.6. Thermometric Titrimetry

Bark and Grime⁶⁷ developed a thermometric assay for several sulphonamides including sulphamerazine. The sulphamerazine was dissolved in the minimum volume of 0.1M aqueous sodium hydroxide solution and the pH adjusted to between 8.0 and 9.18 with 0.1M nitric acid solution. The solution was titrated with standard silver nitrate solution and the data calculated from the resulting enthalpogram. Excipients such as lactose, starch, and magnesium stearate did not interfere. Details of the apparatus required for this assay have been described by Bark and Bate^{68,69}.

Sulphonamides have also been determined by a catalytic thermometric titration technique. The principle of the method is that weak acids are titrated with a base in non-aqueous media using acrylonitrile as a thermometric indicator. Thus, at the endpoint the acrylonitrile undergoes alkali catalysed anionic polymerization with a corresponding evolution of heat which is measured. Greenhow and Spencer⁷⁰ determined sulphamerazine by this technique using dimethylformamide as the non-aqueous solvent and 0.1M or 0.01M tetra-n-butylammonium

hydroxide in methanol-toluene or isopropanol-toluene as titrant. The lower practicable limit of determination was shown to be 0.0001 m.equiv. of drug. Interferences were evident in the presence of acidic excipients.

6.4. Spectrophotometric Assay Procedures

6.4.1. Infrared Spectroscopic Methods

The application of infrared spectroscopy to the quantitative assay of sulphonamides has been of limited interest as reflected by a distinct lack of publications in this field. However, Dolinsky⁷¹ determined sulphamerazine and sulphadiazine in mixture by this technique using carbon disulphide as solvent. Oi and Miyazaki⁷² also determined sulphamerazine in mixture with sulphathiazole using dimethylformamide as solvent.

6.4.2. Ultraviolet Spectroscopic Methods

Ultraviolet spectrophotometry has found some use in the determination of sulphamerazine. Since this drug is normally incorporated into a double or triple sulphonamide formulation the methods most commonly available involve its determination in the presence of one or two other sulphonamides. Marzys^{7,73} described a method for the assay of sulphamerazine in the presence of sulphadiazine and sulphathiazole without prior separation. Following the determination of sulphadiazine by the 2-thiobarbituric acid colorimetric method direct ultraviolet spectrophotometry was used to measure the quantities of sulphamerazine and sulphathiazole. The results were then calculated by solving two simultaneous equations. Using a similar principle Zajac⁷⁴ and Rapaport and Shakh⁷⁵ determined sulphamerazine in formulations with other sulphonamides. The use of a computer programming technique for resolving the ultraviolet spectra of triple sulphonamide tablets containing sulphamera-

zine has been described by Madsen and Robertson⁷⁶.

6.4.3. Colorimetric Methods

A number of colorimetric methods have been described for the determination of sulphonamides which are applicable to sulphamerazine. Probably the most well known is the Bratton and Marshall method⁷⁷ which involves diazotization of the primary amine function with acidic sodium nitrite solution, decomposing the excess nitrite with sulphamic acid followed by coupling the diazo compound with N-(1-naphthyl)-ethylenediamine. In general this method has found its greatest application in the assay of small amounts of sulphonamide following a paper^{78,79} or thin layer^{80,81,82,83} chromatographic procedure.

Other colorimetric methods have been developed, but have not been as widely used as the Bratton and Marshall procedure. Tulus and Guran⁸⁴ developed a method for sulphamerazine and other sulphonamides using the potassium salt of 1,2-naphthoquinone -4-sulphonic acid as the coupling agent. The use of dimethylaminobenzaldehyde for the quantitative assay of sulphamerazine following paper chromatographic separation has been studied by Luise⁸⁵. A colorimetric determination for sulphamerazine in a tablet dosage form using 9-chloro-acridine has been developed by Stewart and co-workers⁸⁶ who found that the results compared excellently with those obtained by the Bratton and Marshall method.

6.5. Chromatographic Procedures

6.5.1. High Performance Liquid Chromatography

Kram⁸⁷ qualitatively studied the behaviour of some 21 sulphonamides by H.P.L.C. Using a stainless steel column packed with spherical siliceous particles coated with a strong anion exchanger the

retention times of the drugs were established using a mobile phase of 0.01M sodium borate containing various levels of sodium nitrate. From these studies the optimum sodium nitrate levels were predicted for the separation of sulphamerazine, sulphadiazine and sulphamethazine, the official trisulphapyrimidines.

A quantitative H.P.L.C. assay for the trisulphapyrimidines has been reported by Poet and Pu⁸⁸ using a "Zipax" SCX(DuPont) cation exchange column with 0.2M disodium phosphate buffer solution(pH 6.0) as the mobile phase. Sulphadimethoxine was chosen as the internal standard. The recommended pressure of 1000 psig produced a solvent flow rate of 0.7-0.8 ml./min. resulting in a 15-20 minute separation time. Analytical data was obtained for four representative lots of tablet formulations and two suspension formulations, the calculated coefficients of variance for replicate injections ranging from 0.9 to 4.0%.

Westlie and co-workers⁸⁹ have developed a liquid-solid chromatographic assay procedure which is applicable to the trisulphapyrimidines. A MicroPak Si-10 column was used in conjunction with a mobile phase consisting of chloroform, methanol, ammonia 25% (365, 75, 10) flowing at a rate of 0.73ml./min. Sulphathiazole was included as an internal standard.

A H.P.L.C. procedure for the separation of a range of sulphonamides utilising silica gel as the column packing has been described by Cobb and Hill^{89A}. The separation was achieved on a 25cm. stainless steel column of internal diameter 4 m.m. packed with Sperisorb S5W 5 μ m diameter spherical silica gel particles. The mobile phase consisted of a mixture of cyclohexane, anhydrous ethanol, glacial acetic acid(85.7, 11.4, 2.9) and the elution was monitored at 260nm using a

Cecil CE 212 variable wavelength detector. Initial separations were obtained using cyclohexane-ethanol mixtures of variable composition and it was found that increasing the ethanol content decreased the observed retention times. The addition of small amounts of acetic acid significantly increased column efficiency without altering resolution. At a flow rate of 2ml./min. the described mobile phase resulted in a 13 minute retention time for sulphamerazine.

The use of high performance ion pair partition chromatography for the separation of sulphonamides has been investigated by Karger and co-workers⁹⁰. Their efforts represented a feasibility study on the separation of 12 sulpha drugs using a silica gel/CT(Reeve Angel) support, a stationary phase consisting of a cationic counterion (tetrabutyl ammonium ion) buffered to a pH of 9.2 and a mobile phase of n-butanol, hexane (25,75). Under these conditions sulphamerazine was shown to have a retention time of 13-14 minutes.

6.5.2. Gas Chromatography

The main gas chromatographic method reported in the literature for the determination of sulphapyrimidines involved an initial hydrolytic step, the resulting volatile 2-aminopyrimidines being measured. Turczan⁹¹ developed such a method for quantitatively assaying the individual sulphonamides, including sulphamerazine, in the official trisulphapyrimidines. Concentrated sulphuric acid was added to the sample and the mixture heated in an oven at 130°C for 1 hour. The solution was made alkaline and 2-amino-4,6-dimethylpyridine added as internal standard. The components were then separated at 150°C on a column packed with 5% SE-30 + 5% Carbowax 20M on Chromosorb W using flame ionization detection. Excellent recoveries were achieved for the trisulphapyrimidines in both synthetic mixtures and several commercial tablet preparations.

Daun⁹² found that the above method was unsuitable for the determination of sulphamerazine in poultry feeds at levels ranging between 0.002 and 0.05%. The method adopted required the preparation of a relatively clean extract of the feed followed by an extraction step using ethyl acetate. The residue remaining after evaporation of the ethyl acetate was methylated with diazomethane and then acylated with heptafluorobutyric anhydride. The acyl derivatives were found to be easily separated on a 10% DC-200 column at 230°C. Detection was achieved by electron capture. No internal standard was used, the results being evaluated by comparing standard and sample peak heights.

Roeder and Stuthe⁹³ developed a gas chromatographic method for the sulphonamides and their N⁴-acetyl metabolites in blood and urine. The method was applicable to sulphamerazine. The sulphonamides were extracted from the blood and urine samples and then methylated with diazomethane. The methyl derivatives were determined using a column of 3% OV 101 on Gaschrom Q with a relative standard deviation of 5% for the free sulphonamides and 7% for the acetyl conjugates.

The simultaneous qualitative analysis of 14 sulpha drugs and their individual quantitative determinations by gas liquid chromatography were performed by Nose and co-workers^{94A} on solutions of dimethylformamide dialkylacetal derivatives of the drugs in acetone. The derivatives could be detected with an electron capture detector with a highly sensitive response following separation using 10% OV-101 on Chromosorb G HP (80-100 mesh), 5% XE-60 on Gas-Chrom Q (80-100 mesh) or 5% OV-225 on Gas Chrom Q (80-100 mesh) at temperatures between 220 and 240°C. However, the retention times for sulphamerazine varied between about 40 to 80 minutes.

6.5.3. Thin Layer Chromatography

A number of thin layer chromatographic methods have been developed for the identification and quantitative analysis of sulphamerazine and related sulpha drugs. Certain details of these methods are summarised in Table 9 and some spot locating reagents are given in Table 11.

Bican-Fister and Kajganovic⁸⁰ recognised the potential of thin layer chromatography as a more rapid technique than paper chromatography for the quantitative assay of triplesulpha containing preparations such as tablets, suppositories, and suspensions. Using a Kieselgel G layer combined with the solvent system chloroform, methanol(90,10) a quantitative separation of sulphamerazine, sulphathiazole and sulphadiazine was achieved. For the separation of certain mixtures of sulphamerazine, sulphacetamide, sulphamethazine and sulphadiazine the solvent system chloroform, methanol, 25% ammonia solution(90,15,2.4) was found to be better. Following elution from the adsorbent the separated sulphonamides were at first assayed by a U.V. method but Bican-Fister and Kajganovic found that the Kieselgel G gave a high contribution to the blank absorbance. They, therefore, applied the Bratton and Marshall colorimetric method, excellent recoveries being obtained for all the sulphonamides previously mentioned. Limits of error for sulphamerazine ranged between $\pm 3.2\%$ to $\pm 4.1\%$ in synthetic mixtures with the other sulphonamides.

Brunner⁸¹ developed a thin layer method for the analysis of trisulphapyrimidine preparations containing sulphamerazine, sulphadiazine and sulphamethazine using silica gel GF plates and a solvent system comprising chloroform, methanol, ammonia(30,12,1). Again the Bratton and Marshall colorimetric method was used resulting in excellent recoveries. A collaborative study⁸² on the use of this method found that the coefficients of variance for the individual compounds ranged from 0.76 to 1.66. The tri-

TABLE 9
Thin layer chromatography of sulphamerazine

<u>Adsorbent</u>	<u>Solvent System</u>	<u>R_f</u>	<u>Use</u>	<u>Ref.</u>
Kieselgel G.	a) Chloroform, methanol (90,10).	-	Quantitative assay for trisulphapyrimidine preparations.	80
Silica gel G: impregnated with fluorescein.	b) Chloroform, methanol, 25% ammonia (90,15,2.5) Chloroform, ethanol, heptane (1,1,1) contain- ing 1.2% water.	0.57	Identity test.	94
Polyamide CM1011.	a) Chloroform, 95% ethanol (90,10).	0.79	Identity test.	95
	b) Ethyl acetate, 95% etha- nol (80,20).	0.83	" "	
	c) Water, 95% ethanol (60,40).	0.59	" "	
Plaster of Paris impregnated with zinc ferrocya- nide.	a) 0.03M aqueous acetic acid.	0.01	Identity test.	96
	b) 1.74M aqueous acetic acid.	0.17	" "	
	c) 3.33M aqueous acetic acid.	0.38	" "	
Silica gel G impregnated with sodium hydroxide.	a) Chloroform, methanol (4,1).	0.56	Identity test.	97
	b) Acetone, methanol (4,1).	0.61	" "	

TABLE 9 (cont'd)

Thin layer chromatography of sulphamerazine

<u>Adsorbent</u>	<u>Solvent System</u>	<u>R_f</u>	<u>Use</u>	<u>Ref.</u>
Silica gel G impregnated with potassium hydrogen sulphate.	Chloroform, carbon tetrachloride, methanol (7,2,1).	0.34	Identity test.	97
Silica gel G.	Ethyl acetate, methanol (9,1).	0.59	Identity test.	97
Silica gel G.	a) Ethyl acetate, methanol, 25% ammonia (17,6,5).	0.47	Identity test.	98
	b) Petroleum ether, chloroform n-butanol (1,1,1).	0.67	" "	
Silica gel.	Chloroform, methanol (95,5).	0.29	Identity test.	98
Silica gel GF.	Ethyl acetate, methanol (9,1).	0.63	Identity test.	99
Silica gel GF.	Chloroform, methanol ammonia (30,12,1).	-	Quantitative assay for trisulphapyrimidine preparations.	81
Silica gel H impregnated with sodium hydroxide.	Chloroform, methanol (9,1).	-	Quantitative assay for feed concentrates or premixes.	100
Silica gel G precoated plates (Analtech).	Acetone, n-heptane, methanol, 28-30% ammonia, n-butanol (72,21,9,10,10).	0.31	Identity test and quantitative assay in animal tissues.	83

TABLE 9 (cont'd)

Thin layer chromatography of sulphamerazine

<u>Adsorbent</u>	<u>Solvent System</u>	<u>R_f</u>	<u>Use</u>	<u>Ref.</u>
Silica gel G.	pH 7.4 aqueous veronal acetate.	-	R _M -structure activity correlation.	101
Polyamide 11.	a) pH 7.4 aqueous veronal acetate.	-	R _M -structure activity correlation.	101
	b) pH 7.4 aqueous veronal acetate containing 10% acetone.	-	"	
Silica gel.	Chloroform, methanol, ammonium hydroxide (30,12,1).	-	Quantitative assay for trisulphapyrimidine tablets and oral suspensions.	102
Silica gel 60 (Merck precoated).	a) Chloroform, ethanol (9,1).	0.33	Quantitative assay in human urine.	128
	b) Chloroform, ethanol, ammonium hydroxide, (8,2,0.1).	0.20		
	c) Chloroform, ethanol, dioxane, acetic acid, (8,1,1,0.1).	0.49		
	d) Ethyl acetate, dioxane, acetic acid (8,2,0.1).	0.46		

sulphapyrimidines have also been assayed by a combined T.L.C. - in situ densitometric method^{82A}.

One of the more common sulphonamide mixtures used in animal therapy contains sulphamerazine with sulphaquinoxaline, sulphathiazole, and sulphamethazine. Cieri¹⁰⁰ showed that the sulphamerazine, sulphamethazine and sulphathiazole contents of these mixtures were best determined by a thin layer method rather than by the gas chromatographic method proposed by Daun⁹² (reviewed in section 6.5.2.). Using silica gel H impregnated with sodium hydroxide and chloroform, methanol (90,10) as the solvent system Cieri assayed the isolated components by an ultraviolet absorption method which allowed the components to be determined within 2-3% of the actual amounts present.

Thin layer chromatography is now the U.S.P.XIX¹⁰² official method for the determination of sulphamerazine, sulphadiazine and sulphamethazine in trisulphapyrimidine tablets and oral suspensions having replaced the paper chromatographic method of the U.S.P.XVIII. The method involves the use of silica gel as adsorbent combined with chloroform, methanol, ammonium hydroxide (30,12,1) as solvent system. The separated sulphapyrimidines are quantitatively determined using the Bratton and Marshall colorimetric procedure.

A thin layer chromatographic screening method for the estimation of sulphamerazine and other sulphonamide residues in poultry tissues has been reported by Philips and Trafton⁸³. The minimum detectable amount of sulphonamide was found to be about 2 µg or 0.04 p.p.m. using a 50g. sample. To determine the reproducibility of the method 0.1 p.p.m. of a series of sulphonamides was added to 50g. portions of liver tissue, then re-isolated and assayed by direct colorimetry and by the proposed thin layer method. The mean recoveries were 88 and 81% respectively. The recover-

ies of sulphamerazine were respectively 91 and 80%.

T.L.C. has also been used for the estimation of sulphamerazine in biological fluids (see section 7) and for the examination of sulphamerazine decomposition products (see sections 5.1 and 5.2).

6.5.4. Paper Chromatography

Paper chromatography was originally used extensively for the separation, identification and quantitative analysis of sulphonamide mixtures. A number of applications are summarized in Table 10 and some spot location agents are given in Table 11.

Sulphamerazine has been quantitatively determined in mixtures with other sulphonamides by a number of workers^{78,79,104-111}. Most methods used Whatman No. 1 paper, the main variation being in the composition of the mobile solvent system. The Bratton and Marshall colorimetric method has been extensively used for the quantitation of the isolated components^{79,103,106-108,111}.

6.5.5. Ion-Exchange and Partition Chromatography

Hutchins and Christian¹¹³ assayed sulphamerazine by an isotope dilution technique after prior separation on an Amberlite IR-120(H⁺) column. Gilmer and Pietrzyk¹⁴⁴ reported the distribution coefficients of several sulphonamides on H⁺-form, macroporous and gel-type resins for a number of water-organic solvent mixtures. A mixture of sulphabenzamide, sulphacetamide, sulphadiazine, sulphamerazine and sulphapyridine was successfully separated by using 40, 52, 64, 77 and 90% dimethylsulphoxide solutions as elutriants.

Selzer and Banes¹¹⁵ reported a column chromatographic method for the separation, detection and estimation of sulphonamide residues in milk. The recovery of sulpha-

TABLE 10

Paper Chromatography of Sulphamerazine

<u>Paper</u>	<u>Ascending or Descending</u>	<u>Solvent System</u>	<u>R_f</u>	<u>Use</u>	<u>Ref.</u>
Whatman No.1	Ascending	Butanol, ammonia water (40,10,50).	0.3	Separation of metabolic pro- ducts from bio- logical materials.	103
Whatman No.1	-	a) Butanol, glacial acetic acid, water (50,15,60). b) Butanol, ammonia, water (40,10,30).	0.50 0.34	Identity test.	104
Whatman No.1 impregnated with 4% aqueous potassium di- hydrogen phosphate	Descending	Butanol saturated with water.	-	Identity test.	105
Whatman No.1	Descending	Butanol, 3% aqueous ammonia (use the or- ganic layer).	0.29	Identity test and quantitative assay.	79
Whatman No.1 impregnated with acetone, formamide (70,30)	Ascending	Chloroform, methyl chloroform (55,5).	-	Quantitative assay for tri- sulphapyrimidines in tablets and or- al suspensions.	106

TABLE 10 (cont'd)
Paper Chromatography of Sulphamerazine

<u>Paper</u>	<u>Ascending or Descending</u>	<u>Solvent System</u>	<u>R_f</u>	<u>Use</u>	<u>Ref.</u>
Whatman No.1 impregnated with acetone, formamide (70,30).	Ascending	Methylene chloride.	-	Quantitative assay for trisulphapyri- midines in tablets and oral suspensions.	107, 111
Whatman No.1	Descending	Butanol, absolute ethanol, 2N ammonia (10,2,4).	0.24	Stability assay.	109
Whatman No.1	Ascending	0.2N aqueous EDTA containing 20% ammonia.	0.87	Identity test.	109
Whatman No.2	Circular	Butanol, acetic acid, water (5,1,4).	-	Identity test and quantitative assay.	110

TABLE 11
Visualization Methods for thin layer and paper
chromatography of sulphamerazine

<u>Reagent</u>	<u>Spot Colour</u>	<u>Reference</u>	
		<u>T.L.C.</u>	<u>P.C.</u>
1. U.V.(254n.m.)-fluorescence quenching .	Dark blue-black.	94	106,107, 108,111
2. Ehrlichs reagent-1% dimethylamino-benzaldehyde + 1-10% conc.HCl in 95% ethanol.	Yellow.	95,97,98	79,105 109,110
3. Bratton and Marshall reagent a)IN HCl, b)5% NaNO ₂ , c)0.1% N-1-naphthyl)-ethylenediamine dihydrochloride.	Reddish-purple.	94,96, 97,98	
4. Copper sulphate -1-5% CuSO ₄ .5H ₂ O in water.	Brown.	97,99	112
5. Fluorescein-1% in acetone, water (3,1), fluorescence-quenching at 254 nm.	Dark blue-black.	97	
6. Copper acetate-saturated solution in methanol.	Brown.	99	
7. Ceric sulphate-2% in water containing 5% conc.H ₂ SO ₄ .	Yellowish-purple.	99	

merazine from milk was found to be 83% at the 0.5 p.p.m. level. Miller¹¹⁶ developed a partition column chromatographic method for the separation and quantitative assay of trisulphapyrimidines. The sulphapyrimidines were quantitatively transferred in acetone to the top of a potassium bicarbonate impregnated Celite 545 column. Sulphamethazine was eluted first using 10% n-butanol in ether saturated with 0.1N aqueous potassium bicarbonate solution. Sulphamerazine was then eluted with 20% n-butanol in ether saturated with 0.1N aqueous potassium bicarbonate and finally sulphadiazine was eluted with 40% n-butanol in ethyl acetate saturated with water. The separated compounds were then assayed by ultraviolet spectrophotometry. When the validity of the method was studied collaboratively several difficulties were encountered with high column blanks which were attributed to the quality of the n-butanol and Celite used. However, the overall results were satisfactory with an overall standard deviation of 2.58%.

Rader¹¹⁷ applied the concept of ion-pairing to the separation of some selected sulphonamides by partition chromatography. One procedure has been applied to the separation of sulphamerazine, sulphamethazine and sulphadiazine by ion-pair formation with the tetrabutylammonium ion followed by separation on a Celite 545 column. The isolated sulphapyrimidines were then quantitatively measured by ultraviolet spectrophotometry.

6.5.6. Electrophoresis

The electrophoresis (400V, 1mA per cm., 15°C, 60 min., developer p-dimethylamino-benzaldehyde) of several sulphonamides¹¹⁸ was studied by Kinoshita and co-workers¹¹⁸ at various pH values adjusted by Clark-Lubs', Sorensen's or Kolthoff's buffer solutions. Sulphamerazine was found to migrate towards the anode. The procedure was unsuitable for the identification of sulphamerazine, sulphaguanidine and sulphadiazine in a ternary mixture. Garber¹¹⁹ has generated

paper electrophoretic mobility data for several sulphonamides, including sulphamerazine, using 1%, 5% and 10% acetic acid as solvent.

6.6. Electrochemical Methods

6.6.1. Polarography

Using polarography coupled with micro-coulometry Okazaki¹²⁰ studied the electrode reactions of several sulphapyrimidines. The optimum conditions for the polarographic reduction of sulphamerazine were determined, a linear plot being obtained of diffusion current against concentration for 0.1-1.0mM solutions of the drug in pH 3.0 and 9.0 aqueous buffers. The reduction was shown to take place within the pyrimidine nucleus by comparison with the polarographic behaviour of 2-aminopyrimidine. Okazaki¹²¹ applied the method to the determination of sulphamerazine in tablets, injectables, syrups and ointments.

Woodson¹²² applied the principles of d.c. and a.c. polarography to the reduction of a number of pharmaceuticals in an aprotic organic solvent system. Using a dropping mercury electrode against a silver wire reference the d.c. half-wave potential of sulphamerazine in acetonitrile - 0.1M tetrabutylammonium perchlorate as solvent system was found to be -1.95v. The corresponding detection limit was 1×10^{-4} moles/litre.

The polarographic behaviour of the Schiff base of sulphamerazine has been studied by Donev¹²³. A linear response to concentration was found and the method was subsequently applied to the determination of sulphamerazine in the blood plasma and urine of animals dosed orally.

6.6.2. Ion Selective Electrodes

Hazemoto and co-workers¹²⁴ constructed an electrode sensitive to sulpha drugs using sulphamerazine and sulphisomidine as

examples. The electrode sensing system consisted of a liquid membrane containing an iron(II)-bathophenanthroline chelate. Rapid and Nernstian responses were exhibited against solutions of sulphamerazine ranging in concentration between 10^{-3} and 10^{-1} M. High selectivity was obtained in the presence of urea, glycine, aminopyrine and p-aminobenzoic acid which are substances known to interfere in the usual colorimetric analysis of sulpha drugs. In contrast small amounts of sodium trichloroacetate and aspirin produced an appreciable effect in the measured potential.

6.7. Bioassay

A method for the microbiological assay of sulphonamides, involving measuring the zone of inhibition of Escherichia coli strain 9 on agar plates, has been developed by Cantelli Forti and Fracasso¹²⁵. A linear plot was obtained for log concentration against inhibition zone diameter along with a sensitivity of 6-50 ug./ml. of 18 sulphonamides tested sulphamerazine was the fifth most active.

Shibata and co-workers¹²⁶ developed a bioassay method for the determination of sulphonamides, including sulphamerazine, using Bacillus megaterium as the challenge organism.

7. Estimation in Biological Fluids

Longenecker¹⁰³ developed a paper chromatographic method for the determination of sulphamerazine in the plasma of chickens fed with a mixture of sulphadiazine, sulphamerazine and sulphathiazole. The blood sample was drawn from the heart and transferred to a test tube containing potassium oxalate. Following centrifugation the plasma was spotted onto Whatman No. 1 paper which was then developed by the ascending technique using a n-butanol, ammonia, water (40,10,50) emulsion as the solvent system. The separated sulphonamides were then located using p-dimethylaminobenzaldehyde re-

agent and ultimately determined using the Bratton and Marshall coupling technique. In the case of blood analysis better separations were achieved when 0.1% of nona-ethyleneglycol monostearate (a nonionic surfactant) was added to the developing solvent. For the analysis of urine the addition of this material was unnecessary.

A horizontal circular paper chromatographic method for the quantitative estimation of sulphamerazine in blood and urine has been developed by Sinha¹²⁷. The chromatograms were run in a circular chromatographic chamber by both the central and lateral flow processes. The drug was located and estimated using p-dimethylaminobenzaldehyde as the colorimetric reagent. The method gave reproducible results.

Ortengren and Treiber¹²⁸ have reviewed the various chromatographic methods available for the estimation of sulphonamides in biological materials. An extension of their report described the quantitative analysis of sulphonamides (including sulphamerazine) and their N⁴-acetyl metabolites in human urine using thin layer chromatography. For a minimum drug concentration of 10 µg./ml. the sample was spotted directly on to the plate. Below this minimum concentration it was necessary to saturate the urine sample with ammonium sulphate followed by extraction with ethyl acetate. The residue from the ethyl acetate layer was then dissolved in a small amount of acetone and spotted on to the plate. The separated sulphonamide and N⁴-acetyl metabolite were ultimately estimated using densitometry.

An extensive review of a quantitative method for the determination of the bacteriostatically active fraction of sulphonamides and the sum of their inactive metabolites in body fluids is given by Rieder^{129, 130}. The Bratton and Marshall colorimetric assay was used for all quantitative measurements. The procedure was applicable to the analysis of blood plasma, serum, interstitial fluid and urine.

Methods for the direct measurement of sulphonamides in biological fluids have been described by Hawking and Lawrence¹³¹.

8. Pharmacology

8.1. Metabolism

Sulphamerazine undergoes three main types of metabolic transformation these being acetylation, glucuronation and hydroxylation.

Acetylation is the most important of these transformations the product being the N⁴-acetyl derivative. The process takes place in the liver to varying degrees in man, monkeys, mice, rats, rabbits and most other animals except dogs. Various aspects of the metabolism of sulphamerazine have been discussed.¹³²⁻¹³⁴

8.2. Absorption, Distribution, Excretion

8.2.1. In Humans

Sulphamerazine is absorbed chiefly from the gastrointestinal tract following oral administration and has a tendency to be more rapidly absorbed than sulphadiazine¹³⁵. Murphy and co-workers¹³⁶ reported certain observations on the absorption, distribution and excretion of sulphamerazine following oral, subcutaneous, intravenous and rectal administration to humans.

Studies on the distribution of sulphamerazine have been described. Iiri¹³⁷ demonstrated the excretion of sulphamerazine into the human parotid saliva, Rumler and co-workers¹³⁸ demonstrated the rapid transport of sulphamerazine across the human placenta, and Boger¹³⁹ studied the extent to which diffusion of sulphamerazine and other sulphonamides into the cerebrospinal fluid depended on their concentrations in the blood. An extensive study of the circulation of sulphonamides, including sulphamerazine, in the human organism has been report-

ed by Alline¹⁴⁰.

A comparison of the renal excretion rates of sulphamerazine and sulphadiazine in human adults with normal renal function has been conducted by Earle¹⁴¹. Sulphamerazine exhibited a lower overall clearance rate indicating extensive reabsorption via the renal tubules and binding to plasma proteins whereas the N⁴-acetyl derivative was excreted rather than reabsorbed. Reinhold and co-workers¹⁴² compared the renal clearances of sulphamerazine and several other sulphonamides in man with that of inulin (non-reabsorbed by the renal tubules).

8.2.2. In Animals

The absorption and excretion of sulphamerazine in mice, rats and monkeys has been studied by Schmidt and co-workers¹⁴³, the results being in good agreement with those obtained by Welch and co-workers¹³⁵ following experiments in animal and human subjects.

Florestano and co-workers¹⁴⁴ compared the blood concentrations produced in dogs, swine and cattle following the parenteral administration of sulphamerazine and several other sulphonamides. The tissue residue depletion of sulphamerazine in sheep has been investigated by Righter and co-workers¹⁴⁵. Prior to this work Lehr¹⁴⁶ demonstrated the distribution of sulphamerazine (in a triple sulphonamide mixture) in the blood, lung and brain of rats and rabbits.

The mechanism of the renal tubular excretory transport of selected sulphonamides has been discussed by Despopoulos and Callahan¹⁴⁷.

8.3. Toxicity

8.3.1. Acute Toxicity

When given orally to white mice as the sodium salt the LD₅₀ of sulphamerazine was about 2.5g./kg., all deaths occurring within 24 hours¹³⁵. Schmidt and co-workers¹⁴³ have discussed the relative toxicities of sulphamerazine, sulphamethazine and sulphadiazine. The oral acute toxicity of sulphamerazine in mice was found to be 3.3g./kg. at a corresponding blood concentration of 148mgm.%. The LD₅₀ of the N⁴-acetyl derivative was 0.7g./kg. at a corresponding blood level of 66 mgm.%.

8.3.2. Chronic Toxicity

Welch and co-workers¹³⁵ have studied the chronic toxicity of sulphamerazine in rats, dogs and monkeys. The comparative chronic toxicities of sulphamerazine, sulphadiazine and sulphamethazine¹⁴³ have been reported by Schmidt and co-workers¹⁴³.

8.3.3. Clinical Toxicity

The various toxic manifestations which have been observed during the clinical use of sulphamerazine include renal damage, acute loin pain, nausea and vomiting, skin rash, fever, leukopenia, thrombocytopenia, and psychosis¹⁴⁸⁻¹⁵⁰. Of all these manifestations the problem of renal damage has received the greatest attention. The more common types of renal damage resulted following the deposition of drug and/or drug metabolite crystals in the kidney and urine (crystalluria). Sulphamerazine itself has been shown to produce renal damage in both animals^{135, 143} and humans¹⁴⁸⁻¹⁵⁰, but as with other sulphonamides the incidence of renal damage has been related to the pH dependent solubility of the drug and its N⁴-acetyl derivative (see section 2.11.1). The administration of an alkali with the drug helped to overcome the problem of crystalluria but Lehr¹⁵¹ pointed out that adequate alkalization cannot always be accomplished in every patient since in

certain cases such as cardiac and renal insufficiency alkalization was contraindicated. The incidence of renal damage was eventually overcome with the advent of the triple sulphonamide formulations^{20,152-155}.

9. Protein Binding

The relationship between the blood levels attained by sulphamerazine and its degree of binding in plasma has been discussed by Gilligan^{155A}. In vitro experiments conducted with pH 7.4 blood plasma containing 10mgm.% of sulphamerazine and 7% of protein revealed that only 16% of the drug was freely diffusible. Beyer and co-workers¹⁵⁶ during studies on the renal elimination of sulphamerazine by the dog showed that at plasma levels of 6 mgm.% the proportion bound to plasma protein was 36.5%. Dialysis and electrophoresis were used by Dessi and Barattini¹⁵⁷ to determine the interaction of sulphamerazine with the serum protein of the rabbit. The factors influencing the degree of binding were the degree of ionization of the drug and the pH of the medium. In vivo, sulphamerazine was found to be bound to the protein to the extent of 3%.

Scholtan¹⁵⁸ showed that the protein - sulphonamide ratio in human and animal serums followed the Freundlich adsorption isotherm. A relation between binding capacity, tissue distribution and curative action was demonstrated.

The interdependence between the elimination by glomerular filtration and plasma protein binding of some sulphonamides¹⁵⁹ was examined by Portwich and co-workers. Protein binding was measured with an ultracentrifuge and the elimination ratio by inulin clearance under tubular blockade. With sulphamerazine, which is resorbed but not secreted, the kidney elimination was found to be dependent on the degree of protein binding.

Moriguchi and co-workers¹⁶⁰ studied the binding of sulphonamides, including sulphamerazine, to bovine serum albumin demonstrating a correlation between binding constant, decreased in vitro bacteriostatic activity and pK_a . In an extension of this work Wada and Moriguchi¹⁶¹ spectrophotometrically evaluated the binding of N⁴-acetyl sulphonamides to bovine serum albumin. Agren and co-workers¹⁶² also showed a correlation between pK_a , pH and binding to human albumin in vitro. The degree of binding of sulphamerazine presented as a function of pH increased from the acidic to the basic side of the pK_a value indicating that the anionic form is more bound than the uncharged species.

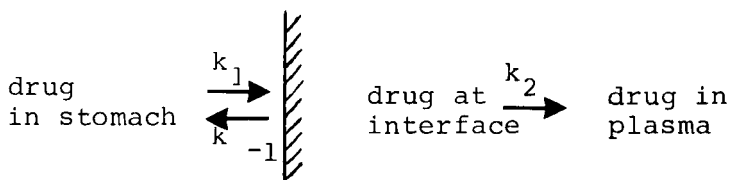
The relationship between structure and binding of sulphonamides to bovine serum albumin was studied by Hsu and co-workers¹⁶³ using a fluorescence probe technique. The work established that the substituent at the N¹-position played an important role in the binding to hydrophobic protein sites. The methyl group at the 4-position within the pyrimidine ring of sulphamerazine apparently significantly increases the binding of the drug to albumin.

Other studies on the binding of sulphamerazine to proteins have been reported¹⁶⁴⁻¹⁶⁸.

10. Pharmacodynamics

The kinetic mechanisms of the absorption of the sulphonamides through the lipidal barrier and the relationship of absorption rates and oil-water partition coefficient has been investigated by Koizumi and co-workers²⁴. An absorption rate vs. pH profile was obtained from experiments in which male rats were orally dosed with solutions of the drug at various pH values. Sulphamerazine exhibited a variable rate of absorption, the rate reaching a maximum at around pH 6-7 and then falling off under more alkaline conditions showing that the unionized form was absorbed predominantly.

However, according to theory the pH at which sulphamerazine was completely unionized was calculated to be 4.7. This discrepancy was attributed to certain characteristics of gastric juice and the site of absorption in the stomach. The absorption rate of the unionized form of f_1 sulphamerazine was found to be 0.07 hr.^{-1} compared to 0.09 hr.^{-1} for sulphamerazine. This and other kinetic data gave a linear correlation with the reciprocal of the partition coefficient determined between isoamyl acetate and water suggesting that the elementary processes of absorption followed the model shown below.



That the hydrophobic interaction between sulphonamides and the intestinal membrane formed an important factor in their absorption¹⁶⁹ was shown by Nogami and co-workers¹⁶⁹. A physico-chemical approach based on the adsorption of sulphonamides from pH 7.4 aqueous solution by carbon black was used as a model. The experiments showed that the introduction of a methyl group into the pyrimidine ring, as in the case of sulphamerazine, not only increased the adsorption on to carbon black but also increased the binding to bovine serum albumin and increased the rate of absorption from the rat small intestine. A good correlation was also obtained between the degree of absorption and the partition coefficient in n-butanol water.

Augustine and Swarbrick¹⁷⁰ used a three-phase model cell employing an isopentyl acetate liquid lipid barrier to test the in vitro transport rates of a series

of N¹-substituted heterocyclic sulphonamides, including sulphamerazine. Correlations were found between the in vitro transport rates (determined as a function of pH), partition coefficients in isopentyl acetate-aqueous buffer, and in vivo gastric, intestinal and rectal absorption data. The studies indicated that the maximum rate of transport occurred at a pH intermediate between the two pK_a values of each drug and that it was related to the fraction of unionized drug.

The use of high performance liquid chromatography for quantitative structure-activity relationships of sulphonamides has been investigated by Henry and co-workers¹⁷¹. The retention volumes for a group of sulphonamides which included sulphadiazine, sulphamerazine and sulphamethazine were obtained in three different H.P.L.C. columns and subsequently correlated with log partition coefficient (n-octanol-water), pK_a, and biological activity.

Taraszk and Forist¹⁷² discussed such kinetic aspects as half lives for absorption and elimination as well as limiting solubilities in connection with the administration of the triple sulphas sulphadiazine, sulphamerazine and sulphamethazine. Two simple hypothetical cases were presented: a) the selection of the ratio of two drugs with different rate constants for absorption and elimination to obtain average asymptotic serum levels of each drug on multiple dose administration and b) the selection of the ratio of two drugs with different rate constants for absorption and elimination, and different solubilities to minimise the risk of crystalluria. The latter was extended to the triple sulphas on the basis of solubility and human blood data giving an optimum ratio of 1:3:4 for sulphadiazine, sulphamerazine and sulphamethazine respectively.

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TRIAMCINOLONE HEXACETONIDE

Vladimir Zbinovsky and George P. Chrekian

CONTENTS

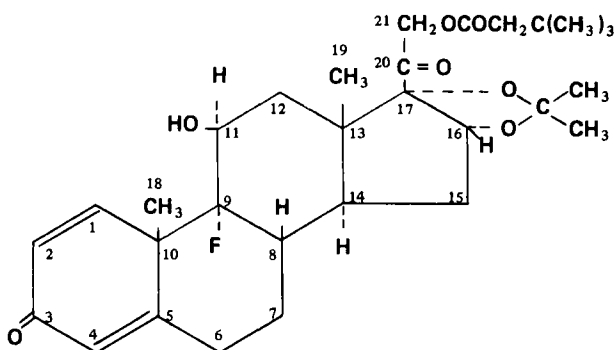
1. Description
 - 1.1 Name, Formula, Molecular Weight
 - 1.2 Appearance, Color, Odor
2. Physical Properties
 - 2.1 Infrared Analysis
 - 2.2 Nuclear Magnetic Resonance Spectrum
 - 2.3 Ultraviolet Spectrum
 - 2.4 Mass Spectrum
 - 2.5 Optical Rotation
 - 2.6 Melting Point
 - 2.7 Thermogravimetric Analysis
 - 2.8 Differential Thermal Analysis
 - 2.9 Solubility
 - 2.10 Crystal Properties
3. Synthesis
4. Stability, Degradation
5. Pharmacodynamic Studies
6. Methods of Analysis
 - 6.1 Elemental Analysis
 - 6.2 Direct Spectrophotometric Analysis
 - 6.3 Colorimetric Analysis
 - 6.4 Polarographic Analysis
 - 6.5 Chromatographic Analysis
 - 6.51 Thin Layer
 - 6.52 Column

Triamcinolone Hexacetonide

1. Description

1.1 Name, Formula, Molecular Weight

Triamcinolone hexacetonide is 9-Fluoro-11 β ,16 α ,17, 21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-acetal with acetone 21-(3,3-dimethyl-butyrate). It is also known as Pregna-1-4-diene-3,20-dione, 21-(3,3-dimethyl-1-oxobutoxy)-9-fluoro-11-hydroxy-16,17-[(1-methylethylidene)bis(oxy)]-, (11 β , 16 α)-.



$C_{30}H_{41}FO_7$

MOL. Wt.: 532.65

1.2 Appearance, Color, Odor

White to cream colored, odorless crystalline powder.

2. Physical Properties

2.1 Infrared Analysis¹

The infrared spectrum of triamcinolone hexacetonide (Lederle House Standard No. 48550-115) is presented in Figure

1. The spectrum was taken in a KBr pellet. The following bands (CM-1) were assigned to triamcinolone hexacetonide:

- a. Characteristic for 21-OAc=0 in the presence of 20-one: 1745
- b. Characteristic for 20-one in the presence of 21 OAc: 1715
- c. Characteristic for α , β unsaturated 3-One: 1664
- d. Characteristic for double bond system, Δ -1, 4: 1618, 1605
- e. Characteristic for C-O stretching bands of 16; 17 acetonide: 1078, 1063
- f. Characteristic for Cis CH of the Δ -1, 4 system: 890

2.2 Nuclear Magnetic Resonance Spectrum¹

The NMR spectrum Figure 2 was obtained by dissolving 40 mg of Lederle House Standard No. 48550-115 in 0.5 ml of deuteriochloroform plus one drop of hexadeutero dimethyl sulfoxide. Tetramethyl silane was added to the solution as internal standard. The spectrum is a single scan on an HA-100D Varian Spectrometer. The spectral assignments of triamcinolone hexacetonide are shown in Table I.

2.3 Ultraviolet Spectrum

The λ max. of the triamcinolone hexacetonide (Lederle House Standard No. 48550-115) is 238 nm, $\epsilon \approx 15,500$.

FIGURE 1

Infrared Spectrum of Triamcinolone Hexacetonide in KBr Pellet;
Instrument: Perkin - Elmer 21

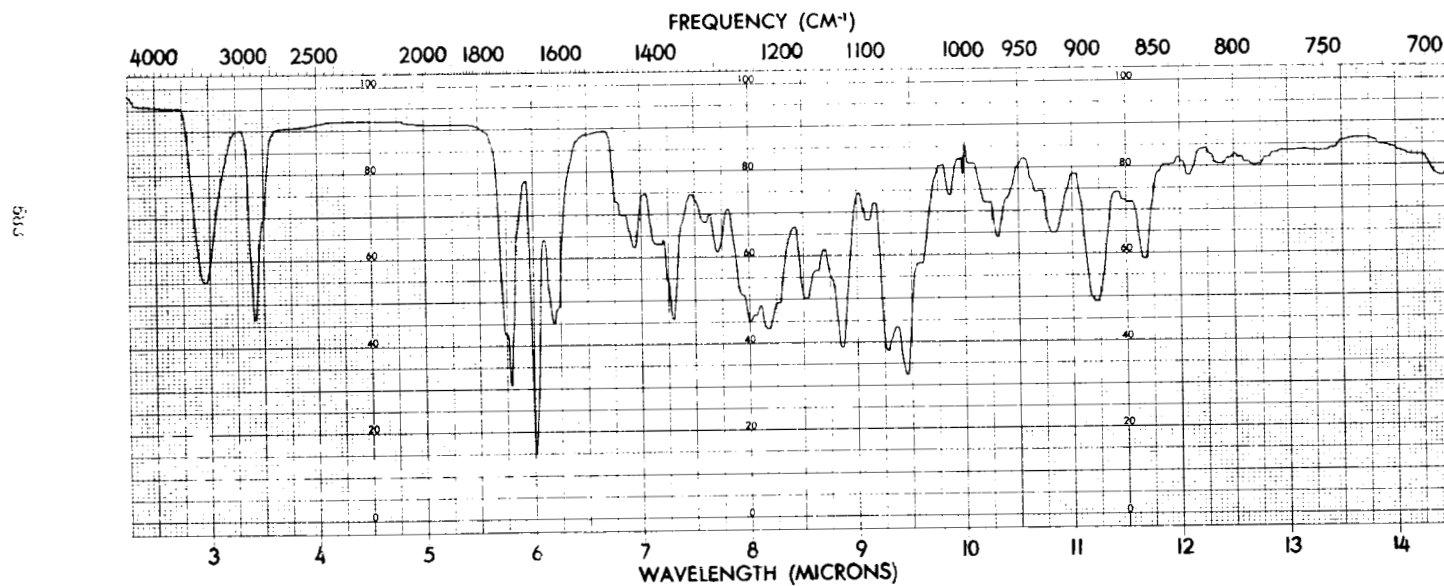


FIGURE 2

NMR Spectrum of Triamcinolone Hexacetonide Containing Tetramethylsilane
as Internal Standard. Instrument: HA-100D

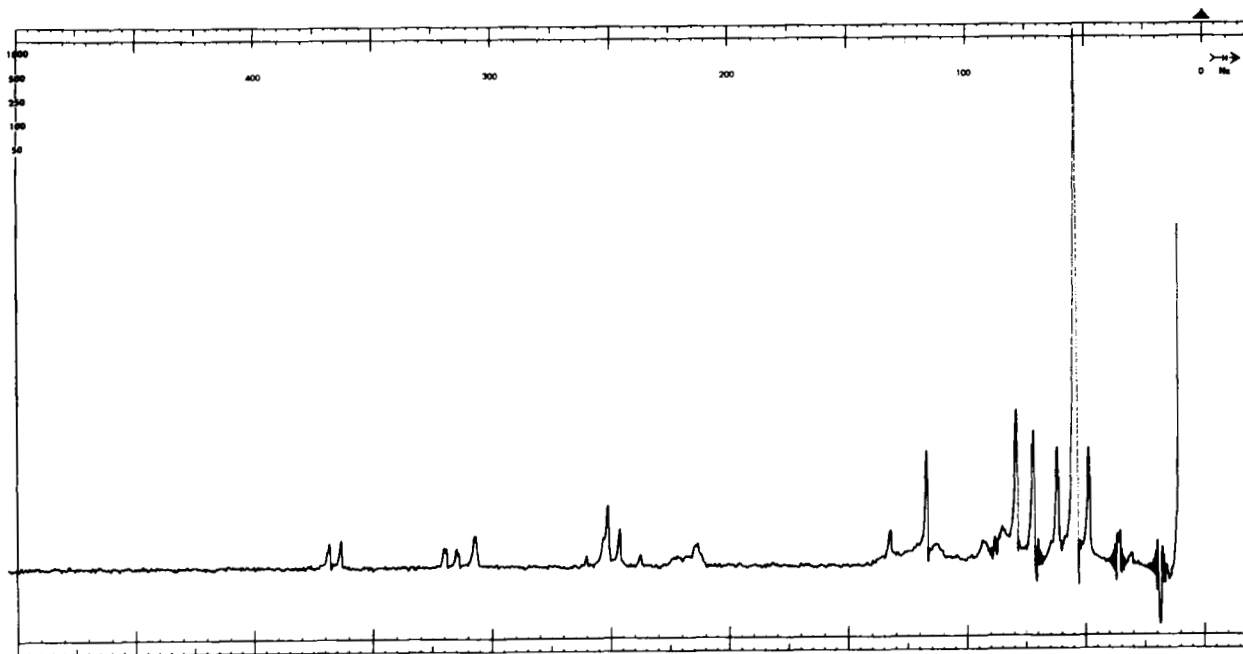


TABLE I

NMR Spectral Assignments of Triamcinolone Hexacetonide

<u>Protons at</u>	<u>Chemical Shift (5)</u>	
C ₁	7.29	d; J _{1,2} = 10.0
C ₂	6.34	dd; J _{1,2} = 10.0, J _{2,4} = 2.0
C ₄	6.14	m
C ₁₁	4.41	m
C ₁₆	5.03	m
C ₁₈	0.97	s
C ₁₉	1.58	s
C ₂₁	4.86	d Jg em = 19
C ₂₁	5.07	d ABq
Acetonide Methyl	1.24	s
Acetonide Methyl	1.44	s
<u>Side Chain at C₂₁</u>		
$\begin{array}{c} \text{O} \quad \text{C} \quad \text{CH}_2 \\ \parallel \\ \text{O} \end{array}$	2.34	s
C(CH ₃) ₃	1.08	s

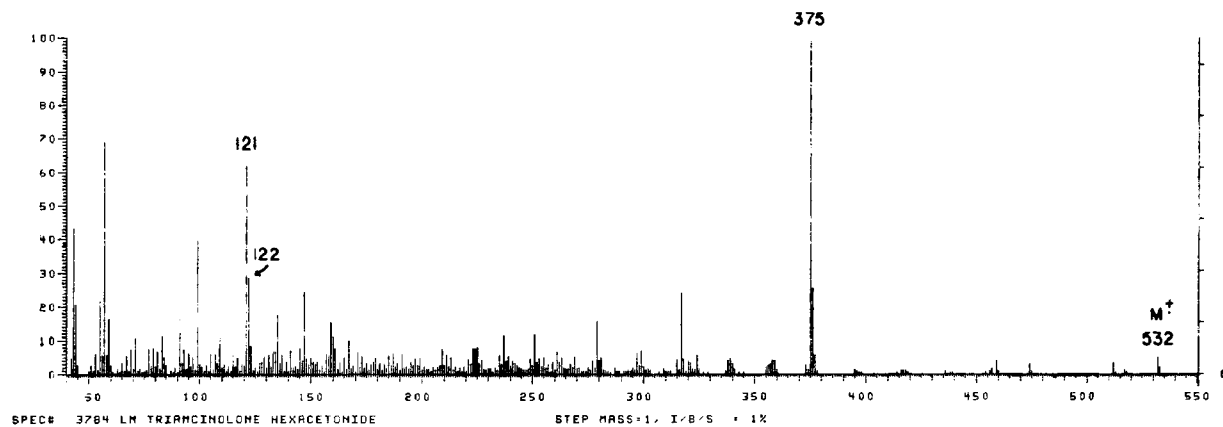
s = singlet; d = doublet; m = multiplet; ABq = AB quartet;
dd = doublet of doublets; J = coupling constant in Hz

2.4 Mass Spectrum¹

The mass spectrum of triamcinolone hexacetonide was run on an AEI MS-9 instrument and is shown in the Figure 3. The molecular ion at m/e 532 is of low intensity. The major fragment ions in the high mass region are observed at m/e 517 (loss of CH₃), 512 (loss of HF) 474 (loss of C₃H₆O), 459 (loss of C₄H₉O). The base peak in the spectrum appears at m/e 375

FIGURE 3

Low Resolution Mass Spectrum of Triamcinolone Hexacetonide.
Instrument: AEI MS-9



and results from cleavage of the bond between C-17 and C-20 with loss of $C_8H_{13}O_3$. Intense ions at m/e 122 and 121 are indicative of a cross conjugated dienone in the A ring.

2.5 Optical Rotation

The optical rotation was determined¹ for triamcinolone hexacetonide in chloroform solution at conc. 1.13%.

$$[\alpha]_D^{25} + 90^\circ \pm 2$$

2.6 Melting Point

The melting point of triamcinolone hexacetonide is $271 - 272^\circ$ (decomposition).

2.7 Thermogravimetric Analysis²

A thermal gravimetric analysis was performed on triamcinolone hexacetonide on a House Standard (No. 48550-115) using a DuPont Model 950 instrument revealed $< 0.2\%$ weight loss up to $180^\circ C$ indicating no significant amount of volatile matter such as water and low boiling organic solvents. The analysis was performed using a nitrogen sweep and a programmed heating rate of $5^\circ C/min$.

2.8 Differential Thermal Analysis²

Differential thermal analysis on triamcinolone hexacetonide (House Standard) using a DuPont Model 990 instrument gave a thermogram displaying a single sharp melting-decomposition endotherm centered at 300° with no indication of any other phase change. The heating rate was programmed at a rate of $10^\circ C/min$.

2.9 Solubility

Solubility determinations at $25^\circ C$ were carried out on Lederle House Standard No. 48550-115 and are presented in Table II.

TABLE II
SOLUBILITY OF TRIAMCINOLONE HEXACETONIDE AT 25°C.

<u>Solvent</u>	<u>mg/ml</u>	<u>% W/V</u>
H ₂ O	0.5	0.050
Hexane	1.3	0.130
Benzene	4.2	0.420
MeOH	6.5	0.650
1-Octanol	7.3	0.730
Ethyl Acetate	7.9	0.790
1-Butanol	11.3	1.130
Abs. Ethanol	11.4	1.140
1-Propanol	11.5	1.150
Dioxane	21.5	2.150
Methyl-Ethyl Ketone	35.4	3.540
Acetone	36.6	3.660
Chloroform	172.6	17.260

2.10 Crystal Properties

Triamcinolone hexacetonide does not form polymorphic forms when recrystallized from solvents used for demonstration of polymorphism in triamcinolone³ and triamcinolone diacetate⁴. Mesley⁵ who inspected triamcinolone acetonide by infrared spectroscopy was not able to demonstrate polymorphic forms in this compound.

The x-ray powder diffraction pattern of triamcinolone hexacetonide⁶ (Lederle House Standard No. 48550-115) is presented in Table III.

TABLE III

POWDER X-RAY DIFFRACTION PATTERN OF TRIAMCINOLONE HEXACETONIDE

<u>d (Å)*</u>	<u>Relative Intensity**</u>
15.70	0.06
13.10	0.13
10.80	0.10
8.80	0.03
7.30	0.07
6.65	0.04
5.90	1.00
5.50	0.01
5.15	0.17
4.75	0.15
4.60	0.01
4.34	0.05
4.13	0.05
3.63	0.07
3.44	0.03
3.32	0.02
3.10	0.12
2.63	0.05
2.58	0.01
2.47	0.03
2.37	0.05
2.14	0.01
2.08	0.02

*d = (interplanar distance) $\frac{n\lambda}{2 \sin \theta}$, $\lambda = 1.539\text{Å}$

**Based on highest intensity of 1.00

Radiation: $K\alpha_1$, and $K\alpha_2$ Copper

3. Synthesis

Triamcinolone acetonide, whose synthesis was described

previously^{8,9} is used as starting material for synthesis of triamcinolone hexacetonide. The synthesis consists of reacting triamcinolone acetonide with tert. butylacetyl chloride in pyridine at +4°C and is shown in Figure 4.

4. Stability, Degradation

Triamcinolone hexacetonide seems to be quite stable in vivo, no enzymatic deacetonization or deesterification was observed and 90% of the compound was excreted unchanged in dogs.¹⁰

Triamcinolone hexacetonide is very stable as a solid. It does not lose its physical appearance and chemical potency when stored at room temperature for more than ten years in an absence of light.

It has been reported¹⁴ that hydrocortisone and prednisolone when exposed to ultraviolet light or ordinary fluorescent laboratory light in alcoholic solution undergo photolytic degradation of the A-ring. Since triamcinolone hexacetonide has the same A-ring as prednisolone it probably also is labile under these conditions.

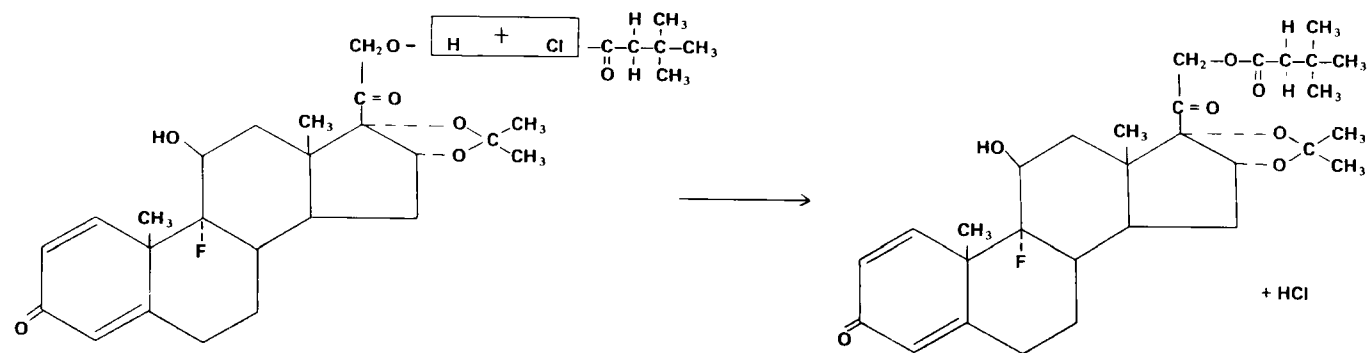
L. L. Smith et al reported¹⁵ that the 21-acetate group in triamcinolone diacetate is easily split off with subsequent oxidation rearrangement and degradation of one side chain in mildly alkaline solution. Since triamcinolone hexacetonide also has an ester group on 21-carbon, it is probable, that this side chain can be easily hydrolysed by the similar mechanism.

5. Pharmacodynamic Studies

In a single intravenous dose of the radioactive triamcinolone hexacetonide administered to the dog, the plasma concentrations of total and ether extractable radioactivity exhibited a biphasic disappearance curve with half lives of about 0.6 to 6 hours for the initial and final phases respectively.¹⁰ Throughout the 7 hour period in which measurable concentration of radioactivity were present, the ratio of plasma to whole blood concentrations was 1.98, indicating little or no penetration to erythrocytes.

In experiments with dogs and cats, less than 10% of the C¹⁴ radioactivity of the oral dose was absorbed and 90% was excreted in feces. No deacetonization or deesterification of triamcinolone hexacetonide was observed and the compound was excreted unchanged. Only small amounts were metabolized into

FIGURE 4.



three more polar, unidentified products.

Intra-articular dose of triamcinolone hexacetonide was released from the site of injection at much slower but steady rate than was the case for triamcinolone acetonide and other related compounds. The half life of radioactivity in this case was about 60 days.

6. Methods of Analysis

6.1 Elemental Analysis for $C_{30}H_{41}FO_7$, Lederle House Standard No. 48550-115²

<u>Element</u>	<u>% Theory</u>	<u>Found</u>
C	67.65	67.79
H	7.76	7.60
F	3.57	3.62

6.2 Direct Spectrophotometric Analysis

The UV absorption maximum at 238 nm has been extensively utilized for assay purposes especially when methanol was used for elution of triamcinolone hexacetonide from thin layer chromatographic plates.¹¹

Triamcinolone hexacetonide has a distinct infrared spectrum, which can be used in qualitative and quantitative analysis.

6.3 Colorimetric Analysis

Blue tetrazolium, the most common reagent used for colorimetric determination of adrenocortical steroids, cannot be applied to triamcinolone hexacetonide, since α -ketol group is not available.

Isonicotinic acid hydrazide (INAH) is used instead to produce yellow derivative of the triamcinolone hexacetonide which has absorption maximum at 380 nm.¹² The color is due to hydrazone formation from $\Delta^{1,4}$ -3 keto group.

6.4 Polarographic Analysis

The polarogram of triamcinolone hexacetonide was obtained by scanning the sample from (-) 0.85 Volts vs. SCE to (-)1.38 Volts vs. SCE using differential pulse mode of operation with full scale range of 3.0V. A single reduction

wave appeared at E_p (-) 1.12 V. vs. SCE, when 0.1M tetrabutylammonium chloride, adjusted to pH 3.5 with phosphoric acid as supporting electrolyte was used. The concentration of triamcinolone hexacetonide was 30 ppm and a well defined peak could be obtained down to 3 ppm. Other parameters for the polarogram, shown in Figure 5 were: modulation amplitude of 50 mV, scan rate 2 mV sec^{-1} , drop rate 1 sec.^{-1} , and a current sensitivity of $2 \mu\text{A}$ full scale.

6.5 Chromatographic Analysis

6.51 Thin Layer

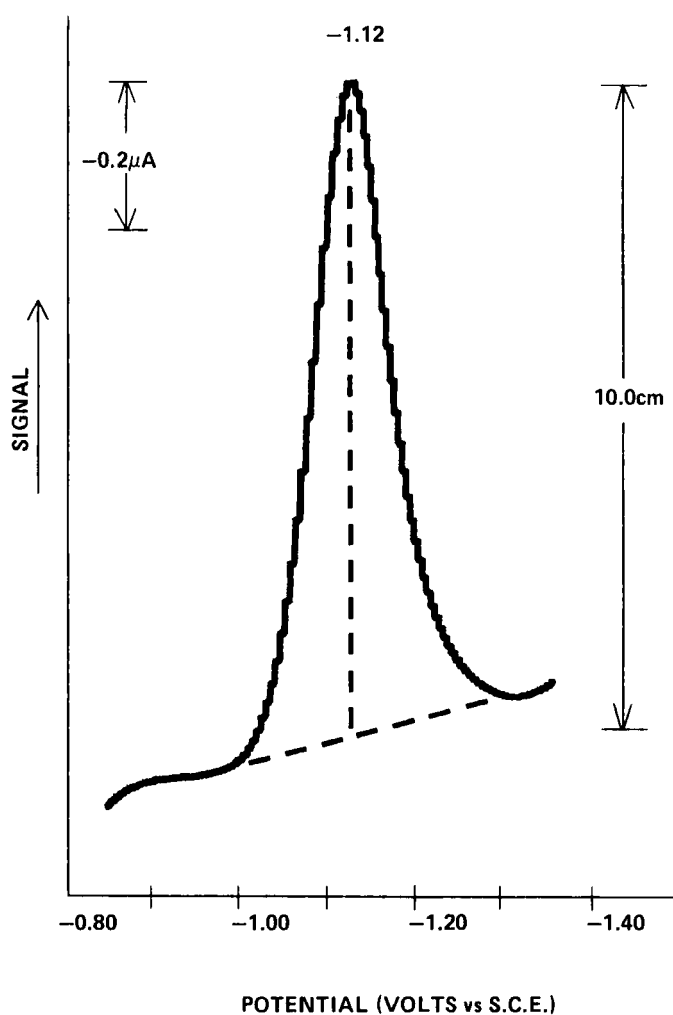
Separation of triamcinolone hexacetonide from 1,2-dihydro triamcinolone acetonide, 1,2-dihydro triamcinolone hexacetonide and triamcinolone acetonide present as minor components has been accomplished by this method.¹¹ Silica Gel GF precoated plates (Analtech Inc.) were used with benzene, Skellysolve C, methanol and p-dioxane-water mixture as developing solvent. Development time was approximately 45 minutes. The approximate R_f values (after rechromatography) were 0.50 for triamcinolone hexacetonide, 0.21 for 1,2-dihydro triamcinolone acetonide, 0.60 for 1,2-dihydro triamcinolone hexacetonide and 0.16 for triamcinolone acetonide. Compounds were eluted with methanol and quantitatively determined spectrophotometrically at 238 nm.

6.52 Column

The Chromatronix Model 3100 instrument was used for High pressure Liquid Chromatography in quantitative determination of triamcinolone hexacetonide in presence of triamcinolone acetonide.¹³ Spherical siliceous packing, was used, employing dichloromethane and isopropanol for the mobile phase. Steroids were eluted and determined at 254 nm. Retention time for triamcinolone hexacetonide was 3.5 min.; triamcinolone acetonide can be eluted in 18 min.

When the measured peak areas and/or peak heights of standards were plotted, a linear relationship resulted between areas or heights and concentration.

Fig. 5. Differential Pulse Polarogram
of Triamcinolone Hexacetonide in 0.1M
Tetrabutylammonium Chloride buffer, pH 3.5



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ADDENDA AND ERRATA

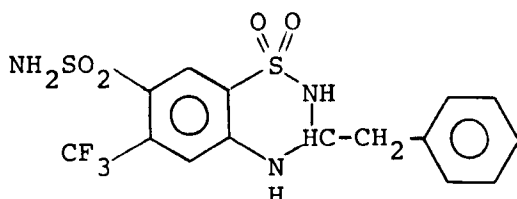
Affiliations of Editors and Contributors

Volume 5, p. vii

Correct affiliation:

Z.L. Chang, Abbott Laboratories,
North Chicago, IllinoisBendroflumethiazide

Volume 5, p. 13

Fig. 6, Correct formula for bendroflumethiazide
(I)

Volume 5, p. 16

Add Section 6.53: Column Chromatographic
Analysis.A column chromatographic method, using a
sodium carbonate column and chloroform-
acetic acid (98+2) and U.V. readout has
been described by F. R. Fazzari, Journal
of the A.O.A.C., 59, p. 96 (1976).Propoxyphene Hydrochloride

Volume 1, p. 316

Add Section 4.7: HPLC Analysis

An HPLC method for tablets and capsules
has been described by R. K. Gilpin,
J. A. Korpi and C. A. Janicki, J. Chromat.,
107, p. 115 (1975).

CUMULATIVE INDEX

Italic numerals refer to Volume numbers.

- Acetaminophen, 3, 1
- Acetohexamide, 1, 1; 2, 573
- Alpha-Tocopheryl Acetate, 3, 111
- Amitriptyline Hydrochloride, 3, 127
- Amphotericin B, 6, 1
- Ampicillin, 2, 1; 4, 517
- Bendroflumethiazide, 5, 1; 6, 597
- Betamethasone Dipropionate, 6, 43
- Cefazolin, 4, 1
- Cephalexin, 4, 21
- Cephalothin Sodium, 1, 319
- Cephadrine, 5, 21
- Chloral Hydrate, 2, 85
- Chloramphenicol, 4, 47, 517
- Chlordiazepoxide, 1, 15
- Chlordiazepoxide Hydrochloride, 1, 39; 4, 517
- Chloroquine Phosphate, 5, 61
- Chlorprothixene, 2, 63
- Clidinium Bromide, 2, 145
- Clonazepam, 6, 61
- Clorazepate Dipotassium, 4, 91
- Cloxacillin Sodium, 4, 113
- Cyclizine, 6, 83
- Cycloserine, 1, 53
- Cyclothiazide, 1, 66
- Dapsone, 5, 87
- Dexamethazone, 2, 163; 4, 518
- Diatrizoic Acid, 4, 137; 5, 556
- Diazepam, 1, 79; 4, 517
- Digitoxin, 3, 149
- Diethyl Sodium Sulfosuccinate, 2, 199
- Diperodon, 6, 99
- Diphenhydramine Hydrochloride, 3, 173
- Disulfiram, 4, 168
- Echothiophate Iodide, 3, 233
- Ergotamine Tartrate, 6, 113
- Erythromycin Estolate, 1, 101; 2, 573
- Estradiol Valerate, 4, 192
- Ethynodiol Diacetate, 3, 253
- Fenoprofen Calcium, 6, 161
- Flucytosine, 5, 115
- Fludrocortisone Acetate, 3, 281
- Fluorouracil, 2, 221
- Fluphenazine Enanthate, 2, 245; 4, 523
- Fluphenazine Hydrochloride, 2, 263; 4, 518
- Gluthethimide, 5, 139
- Halothane, 1, 119; 2, 573
- Hydroxyprogesterone Caproate, 4, 209
- Iodipamide, 3, 333
- Isocarboxazid, 2, 295
- Isoniazide, 6, 183
- Isopropamide, 2, 315
- Isosorbide Dinitrate, 4, 225; 5, 556
- Kanamycin Sulfate, 6, 259
- Ketamine, 6, 297
- Levarterenol Bitartrate, 1, 49; 2, 573
- Levallorphan Tartrate, 2, 339
- Levodopa, 5, 189
- Levothyroxine Sodium, 5, 225
- Meperidine Hydrochloride, 1, 175
- Meprobamate, 1, 209; 4, 519
- Methadone Hydrochloride, 3, 365; 4, 519
- Methaqualone, 4, 245, 519
- Methotrexate, 5, 283
- Methyclothiazide, 5, 307
- Methpyrion, 2, 363
- Metronidazole, 5, 327
- Minocycline, 6, 323
- Nitrofurantoin, 5, 345
- Norethindrone, 4, 268
- Norgestrel, 4, 294
- Nortriptyline Hydrochloride, 1, 233; 2, 573
- Nystatin, 6, 341
- Oxazepam, 3, 441

CUMULATIVE INDEX

- | | |
|---|--|
| <p>Phenazopyridine Hydrochloride, 3, 465
 Phenelzine Sulfate, 2, 383
 Phenformin Hydrochloride, 4, 319; 5, 429
 Phenoxymethyl Penicillin Potassium, 1, 249
 Phenylephrine Hydrochloride, 3, 483
 Piperazine Estrone Sulfate, 5, 375
 Primidone, 2, 409
 Procainamide Hydrochloride, 4, 333
 Procarbazine Hydrochloride, 5, 403
 Promethazine Hydrochloride, 5, 429
 Proparacaine Hydrochloride, 6, 423
 Propiomazine Hydrochloride, 2, 439
 Propoxyphene Hydrochloride, 1, 301;
 4, 519; 6, 598
 Propylthiouracil, 6, 457
 Reserpine, 4, 384; 5, 557
 Rifampin, 5, 467
 Secobarbital sodium, 1, 343
 Spironolactone, 4, 431
 Sodium Nitroprusside, 6, 487
 Sulphamerazine, 6, 515</p> | <p>Sulfamethoxazole, 2, 467; 4, 520
 Sulfasalazine, 5, 515
 Sulfisoxazole, 2, 487
 Testolactone, 5, 533
 Testosterone Enanthate, 4, 452
 Theophylline, 4, 466
 Tolbutamide, 3, 513; 5, 557
 Triamcinolone, 1, 367; 2, 571; 4, 520, 523
 Triamcinolone Acetonide, 1, 397; 2, 571;
 4, 520
 Triamcinolone Diacetate, 1, 423
 Triamcinolone Hexacetonide, 6, 579
 Triclobisium Chloride, 2, 507
 Triflupromazine Hydrochloride, 2, 523;
 4, 520; 5, 557
 Trimethaphan Camsylate, 3, 545
 Trimethobenzamide Hydrochloride, 2, 551
 Tropicamide, 3, 565
 Tybamate, 4, 494
 Vinblastine Sulfate, 1, 443
 Vincristine Sulfate, 1, 463</p> |
|---|--|

A
B 7
C 8
D 9
E 0
F 1
G 2
H 3
I 4
J 5